The JOURNAL of the National Cancer Institute

VOLUME 1 August 1940 No. 1

# THE FEDERAL CANCER CONTROL PROGRAM

By CARL VOEGTLIN, *Chief*, and R. R. SPENCER, *Assistant Chief, National Cancer Institute, National Institute of Health, United States Public Health Service*

Cancer is a unique disease. Knowledge acquired in recent decades has set it apart from all other pathological conditions. It is so strikingly different from the communicable diseases that our present methods of approach to its study demand essentially new laboratory tools and a staff of research workers trained in a variety of scientific fields. Indeed, cancer is not a disease at all in the more restricted use of the term, but rather an abnormal type of cellular growth induced and maintained by means as yet little understood.

## PREVALENCE

In recent years, the number of deaths from cancer reported in the United States has steadily increased. There are, however, good reasons to believe that this increase is due, in part, to a continued improvement in diagnostic methods and to the fact that the older age groups of the population (the cancer ages) have increased.

Recent evidence collected by the National Cancer Institute suggests that there are certain sites, notably the respiratory system, buccal cavity, gastrointestinal tract in both males and females, and the female breast, which show an increase in the adjusted mortality rates for these sites. The complexity of modern life and the greater exposure to various irritating substances in industrial communities may be a contributing factor. In fact, the incidence of cancer in the industrial centers of the Northeast is higher than in the rest of the country. On the other hand, cancer of the skin is far more prevalent in the South and Southwest.

## CHARACTERISTICS OF THE CANCER PROCESS

The cancer process has a much wider biological significance than that of most other diseases. We list briefly here certain fully confirmed facts which support this statement:

(1) It has been found in nearly all mammals, and yet cancer arising in one species can be transferred to an individual of another species only with extreme difficulty and under highly artificial conditions. Even in individuals of the same species, a transplanted cancer will seldom grow and develop. However, when animals are inbred by brother-and-sister mating for many generations and thus the genetic constitution of the individuals becomes identical (homozygous), like true twins, then cancers arising in such a group of animals are readily transplantable among these genetically "pure" lines.

One sees, therefore, that while cancer occurs among so many different species, any given cancer is, paradoxically, not only species specific, but practically individually specific.

(2) Factors contributing to the causation of this abnormal process of cell growth that we call cancer are now known to be multiple; hence the cancer process cannot be compared to the infectious disease process that has a single known

causative agent. Human cancer is definitely not communicable.

(3) Both clinical and experimental evidence point to the fact that chronic irritation plays a part in the causation of cancer.

(4) Numerous chemical substances (various coal-tar products) and many physical agents (heat, light, radium, and X-rays) have been repeatedly proven to initiate the cancer process in experimental animals. The exact mechanism of how these agents act upon the tissue cells, however, is still unknown.

(5) Once the process has started, the cancer-producing agents are no longer necessary to the growth and spread of the cancer—again a striking contrast to the usual infectious process.

(6) Cancer cells are known to be permanently altered. They have been kept growing for more than 12 years outside the animal body (tissue-culture technique) and yet still retain the power to produce a cancer when transplanted into the same strain of animal from whence the cancer cells were originally obtained.

(7) The hormones or internal secretions of the body also play a part in the cancer process. The ovarian hormone, estrone, will induce invariably cancer of the breast in the females of certain strains of mice.

(8) While differences in nuclear structure and chromatin content are demonstrable, as a rule, between normal and cancer cells, no specific differences have been observed in chemical composition, enzyme content, or metabolism.

## THE CANCER-CONTROL MOVEMENT

Our present knowledge of the cancer process, briefly outlined above, not only sets the cancer problem apart but gives us some insight into the complexity of the problem and at the same time indicates the line of attack that future investigations must take.

While this knowledge was being acquired through clinical and laboratory studies, the cancer-control movement had been steadily gaining momentum. It was motivated largely by the realization that approximately one-third of the 150,000 people who die of cancer each year in the United States could be saved if knowledge already in existence could be made available to all of our people and were acted upon promptly by them. Essentially, what this knowledge teaches us about cancer prevention can be summed up in one sentence: "*When* cancer is treated is more important than *how* it is treated, provided the patient is in competent hands."

Twenty-six years ago, professional and lay groups joined to launch in the United States this movement which had as its objective the education of physicians and the public to an effective understanding of the primary importance of early diagnosis and prompt expert treatment of cancer. Initiated by the American Society for the Control of Cancer, and supported by the American Medical Association and the American College of Surgeons, the movement has succeeded beyond all expectations. The Women's Field Army now has well-organized units with commanders and subordinate personnel in 46 of the 48 States and the District of Columbia. Twenty-three thousand voluntary workers have distributed more than 4 million pieces of literature giving information about cancer.

## THE NATIONAL CANCER INSTITUTE ACT

When our knowledge of a disease process is incomplete and when no specific remedy is available, education alone is not enough. Research must continue. Furthermore, the cost of adequate modern cancer therapy is very large. Expensive tools, special equipment, organized services, and physicians highly trained in tumor pathology, radiology, and surgery are the essentials.

For these reasons and because so many of the patients are unable to meet the cost of diagnosis and treatment, cancer has become one of our major public health problems.

The growing anticancer movement in the

United States was climaxed, we believe, in the passage by the Seventy-fifth Congress of the National Cancer Institute Act, which was signed by the President on August 5, 1937. Ninety-six members of the United States Senate attached their names to the bill creating the National Cancer Institute as a division of the United States Public Health Service. Never in the history of the Republic had the entire membership of either body of Congress sponsored a piece of legislation. Obviously, the country has now recognized that the fight against cancer, like that against mental diseases, tuberculosis, and syphilis, has passed from a local and State problem and become a national one.

## THE NATIONAL ADVISORY CANCER COUNCIL

Under the provisions of the National Cancer Institute Act, a National Advisory Cancer Council is created consisting of six members, selected from the ranks of the leading medical and scientific authorities, who are outstanding in the study, diagnosis, or treatment of cancer in the United States. The Surgeon General of the United States Public Health Service acts as chairman of the council, ex officio. The membership of the first council, besides Surgeon General Thomas Parran, consisted of Dr. Ludvig Hektoen, executive director; James Conant, president of Harvard University; Arthur H. Compton, professor of physics, University of Chicago; C. C. Little, director, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine; Francis Carter Wood, director, Institute of Cancer Research, Columbia University; and James Ewing, director, Memorial Hospital, New York City. The membership of this council changes, since the term of office of two of its members expires each year. At the end of the first year, November 1938, Dr. James Ewing and Dr. Francis Carter Wood were succeeded by Dr. James B. Murphy of the Rockefeller Institute for Medical Research, and Dr. Mont R. Reid, professor of surgery, University of Cincinnati, Cincinnati, Ohio. At the end of the second year, November 1939, Dr. C. C. Little and Dr. Ludvig Hektoen were succeeded by Dr. Max Cutler, director, Chicago Tumor Institute, and Dr. George M. Smith, scientific advisor of the Jane Coffin Childs Fund and the Anna Fuller Fund, Yale University School of Medicine. Dr. Hektoen, who had contributed so much to the organization of the Federal program, was retained as a consultant on part-time duty.

The council is authorized by the act:

> (a) To review research projects or programs submitted to or initiated by it relating to the study of the cause, prevention, or methods of diagnosis and treatment of cancer * * *;
>
> (b) To collect information as to studies which are being carried on in the United States or any other country as to the cause, prevention, and methods of diagnosis and treatment of cancer, * * * and * * * make available such information through the appropriate publications for the benefit of health agencies and organizations (public or private), physicians, or any other scientists, and for the information of the general public;
>
> (c) To review applications from any university, hospital, laboratory, or other institution, whether public or private, or from individuals, for grants-in-aid for research projects relating to cancer, and certify to the Surgeon General its approval of grants-in-aid in the cases of such projects which show promise * * *;
>
> (d) To recommend to the Secretary of the Treasury for acceptance conditional gifts pursuant to section 6 * * *.

Under the guidance of Surgeon General Parran, and with the advice of the National Advisory Cancer Council, one of the first steps in the Federal anticancer campaign was to solicit the cooperation of representatives from the American Society for the Control of Cancer, the American College of Surgeons, heads of various foundations and deans of various medical

schools. These men were brought to Washington on December 13, 1937, for a frank discussion of the problem of cancer. Upon the recommendation of this group, a special committee was appointed by the Surgeon General to discuss the fundamental aspects of the cancer problem as well as the fundamental principles of cancer research and to suggest various lines of work which merit investigation. The report of this committee appeared in the Public Health Reports of December 2, 1938. Here the cancer problem has been clarified and formulated in such a manner that it has served as an excellent guide to investigators.

## TRAINING IN DIAGNOSIS AND TREATMENT

Section 5 (b) of the National Cancer Institute Act authorizes the Surgeon General:

> To provide the necessary facilities where training and instruction may be given in all technical matters relating to diagnosis and treatment of cancer to such persons as in the opinion of the Surgeon General have proper technical training and shall be designated by him for such training * * *.

Young physicians who are interested in cancer work as a career and who have had certain basic training may apply to the National Cancer Institute for financial assistance in obtaining special training offered by designated training centers in tumor pathology, radiology, and surgery.

Physicians eligible for training must be graduated from an approved medical school, must have completed at least 1 year of internship in an approved hospital, and must be less than 40 years of age. Appointments are made by the Surgeon General for 1 year and are subject to renewal if the trainee's work is satisfactory to the training center and to the National Cancer Institute. During the past 2 years (1938 and 1939), 38 physicians have received appointments.

Twenty-seven institutions in the United States have been designated as centers where training and instruction may be given under a National Cancer Institute "traineeship," in all technical matters relating to the diagnosis and treatment of cancer. These institutions include at least 14 of our leading schools of medicine, and 6 outstanding hospitals devoted exclusively to tumor and cancer patients.

## COOPERATION WITH STATES

The National Cancer Institute Act authorizes and directs cooperation with State health agencies in the prevention, control, and eradication of cancer. Accordingly, there was established an office in the National Cancer Institute which studies State cancer-control legislation and activities and provides consultation service to health agencies requesting such aid.

The programs of the various States vary in scope. In general, activities may be classified under the following broad headings: Provision of free or part-pay State aid for diagnosis and treatment of cancer patients, free tissue diagnosis, lay and professional education, and statistical research. Some States participate in all of these activities, while others carry on only one or two of them.

Figure 1 shows the 25 States with programs in June 1940. Over half of these programs have been inaugurated since 1937 when the National Cancer Institute was established.

Administration of programs is by State health departments in all instances except three. Nine States have specific cancer laws, while the remainder operate by authority provided in basic health legislation.

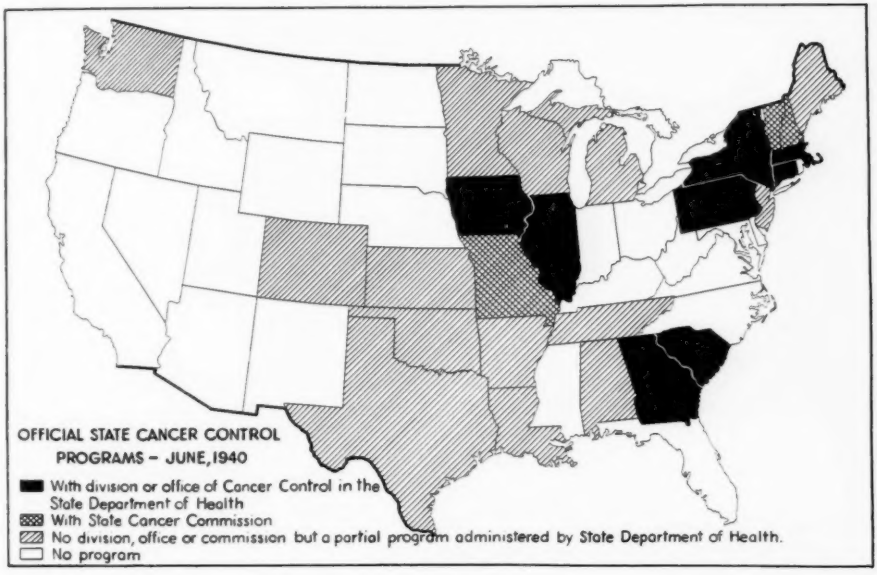

OFFICIAL STATE CANCER CONTROL
PROGRAMS – JUNE, 1940
With division or office of Cancer Control in the State Department of Health
With State Cancer Commission
No division, office or commission but a partial program administered by State Department of Health.
No program

FIGURE 1.

## POLICY REGARDING INVESTIGATION OF "CANCER CURES"

Many claims have been made and still are being made with respect to cures for cancer. Old preparations long ago discarded are offered to the public under new names. Some of these preparations, such as certain salves and pastes, may destroy external cancer, but they cannot destroy a cancer that has spread to the internal parts of the body, and are often more destructive than necessary.

As yet no drug or preparation of any kind has been found that can cure all forms of cancer. The most successful treatment for cancer today involves the removal of the cancer by surgery or destruction of the cancer by X-ray, radium, or electrocautery. In many cases X-ray or radium is combined with surgery. In any case complete removal or destruction can be hoped for only in case the cancer is subjected to treatment in its early and local stages. In the advanced stages the best that can be done (in most cases) is to check in some degree the course of the disease, ameliorate the symptoms, and prolong life. At the present time the diagnosis and treatment of cancer require organized cooperative services—pathological, surgical, and radiological—and special equipment. "Cancer is no longer a one man's job."

The National Cancer Institute has not adopted rigid rules in regard to support of search for new ways of treating cancer or in regard to the approval of testing of treatments and "cures" advocated for cancer. Each formal application for aid is referred to the National Advisory Cancer Council where it will be considered on its merits. Obviously serious attention can-

not be given to requests for testing new forms of treatment unless the following requirements are met:

(1) The method of treatment must be explained fully. There must be no secrecy whatsoever in regard to the composition or the nature of the treatment.

(2) Complete clinical records must be submitted of a suitable number of cancer patients, treated with the remedy or method in question under competent medical supervision, and in each such case the diagnosis of cancer must rest on competent and verifiable microscopic examination.

In answer to inquiries the National Cancer Institute will furnish any factual information in its possession in regard to so-called cancer cures and to the established methods of treating cancer.

## THE LOAN OF RADIUM

Under the provisions of the National Cancer Act, the Surgeon General of the United States Public Health Service is authorized to purchase radium and to make it available on loan to institutions including hospitals, for cancer research, or for the actual treatment of cancer.

In 1938, 9½ gm. of radium, including the platinum-iridium needles, cells, and cylinders, were purchased at a cost of $200,000. The entire amount has been allocated to 48 hospitals and cancer clinics (fig. 2). As funds become available, more radium will be purchased. It should be understood, however, that radium is not loaned unless an institution can meet certain requirements as to qualifications of the personnel, equipment, and organization. The importance of this function of the Institute can be appreciated readily when we consider the opinion of some to the effect that, when the act was passed, the United States possessed only about 50 percent of the radium needed for research and treatment of cancer. It has been

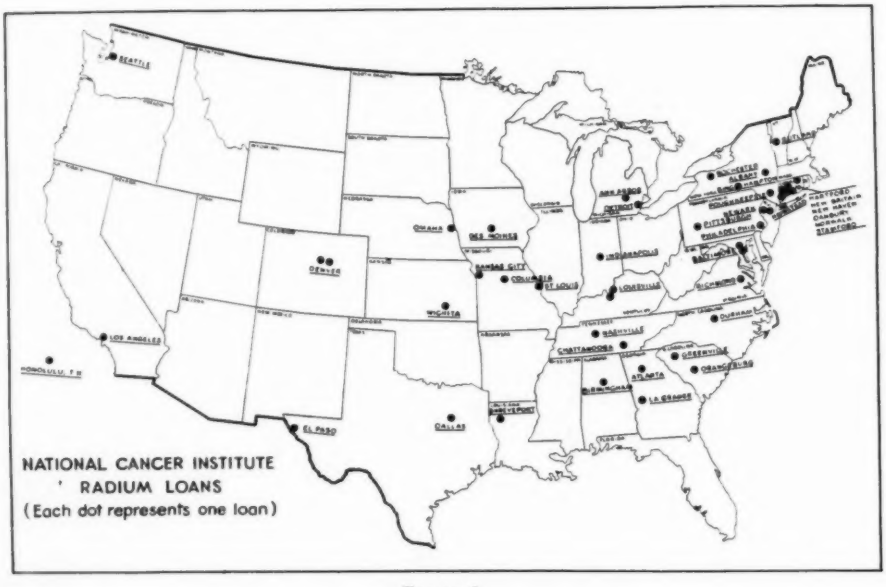



FIGURE 2.

estimated that the entire country has about 200 gm. of radium for medical purposes.

## GRANTS-IN-AID

Upon the recommendation of the National Advisory Cancer Council grants-in-aid amounting to $220,000 have been paid to 19 institutions and individuals since the passage of the National Cancer Institute Act. Thus, in augmenting research and supporting worth-while and promising cancer studies already being carried on by other agencies, the National Cancer Institute has assumed at once a place of the first rank among the agencies and foundations that support cancer-research activities, in addition to the broad frontal attack upon the problem by the Institute's own staff.

## RESEARCH PROGRAM OF THE INSTITUTE

At the laying of the cornerstone of the National Cancer Institute, June 24, 1939, Surgeon General Parran said:

> Because of the vision and foresight of our National Congress, and the generosity of a private citizen, Mrs. Luke Wilson, this beautiful building now stands before you on an imposing site. It is a matter of pride and gratification that we can say today: Nowhere in the world will you find a single structure the equivalent of what is here provided, in physical equipment and facilities, solely for scientific research in a specialized field of medicine * * *. This institution will ever remain a symbol of the faith imposed by the American people in their Federal health agency—the United States Public Health Service. That faith is not founded upon the impressive aspects of splendid buildings, but rather upon an unbroken line of achievement stretching back 141 years to the establishment of the Marine Hospital Service in 1798. Indeed, we of the Public Health Service can say with pride and reverence that this institution—this National Cancer Institute—has been made possible by the splendid work of our pioneer investigators, living and dead.

The building was occupied in October 1939.

249431 – 40——2

It is not generally recognized that cancer research requires years of training and experience. There is a scarcity of investigators who, possessing a thorough training in one of the fundamental sciences, have also acquired a sufficient knowledge of cancer. It was fortunate, therefore, that the Public Health Service for some years preceding the passage of the National Cancer Institute Act had carried on cancer research. One group was working at the National Institute of Health, and the other group at Harvard University. These two groups were moved to the new building. In addition there were appointed, under section 5 (c) of the act a number of research fellows from medical schools and research institutes. On July 1, 1940, the staff included 39 scientists with a wide variety of training and about an equal number of clerical and technical personnel. The organization chart (fig. 3) shows the major scientific disciplines represented on the staff of the Institute. The comprehensive research program includes the following major studies: (1) *Lung cancer*, with respect to its histogenesis, genetic, and chemical predisposing factors; (2) *skin cancer*, in its relation to sunlight (ultraviolet light), air dust, skin secretion, and skin pigments as causative or protective factors; (3) *gastric cancer*, induced by the administration of chemical carcinogens and other agents, and its possible causation by certain diets; (4) *genetic studies*, with particular reference to the nature of the factor present in the milk of certain inbred strains of mice which is a controlling factor of the incidence of breast cancer in mice; (5) *synthesis and testing of chemicals related to natural steroids* for their carcinogenic action; (6) *mode of action of cancer-producing chemicals* as studied on the lower forms of life, on cultures of mammalian cells, and on the higher animals; (7) *therapeutic studies*, partly in col-

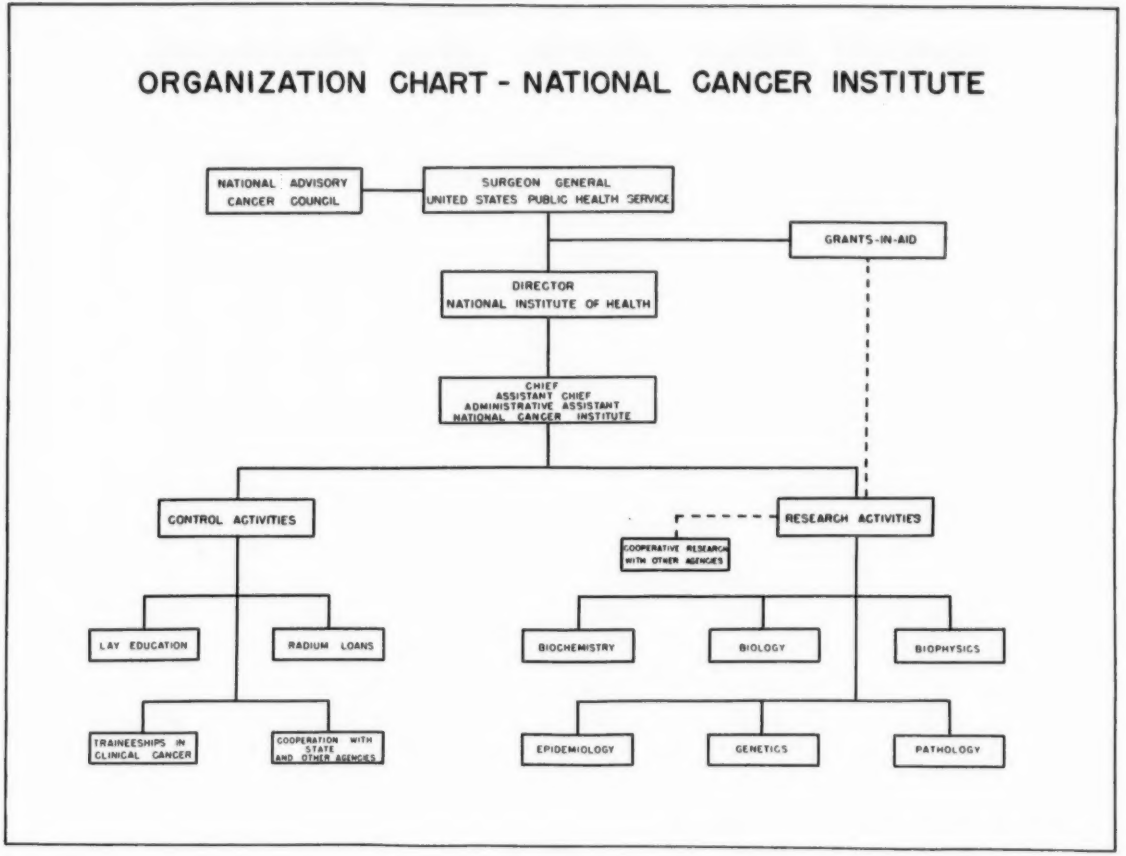

ORGANIZATION CHART - NATIONAL CANCER INSTITUTE
NATIONAL ADVISORY CANCER COUNCIL
SURGEON GENERAL UNITED STATES PUBLIC HEALTH SERVICE
GRANTS-IN-AID
DIRECTOR NATIONAL INSTITUTE OF HEALTH
CHIEF
ASSISTANT CHIEF
ADMINISTRATIVE ASSISTANT
NATIONAL CANCER INSTITUTE
CONTROL ACTIVITIES
RESEARCH ACTIVITIES
COOPERATIVE RESEARCH WITH OTHER AGENCIES
LAY EDUCATION
RADIUM LOANS
BIOCHEMISTRY
BIOLOGY
BIOPHYSICS
TRAINEESHIPS IN CLINICAL CANCER
COOPERATION WITH STATE AND OTHER AGENCIES
EPIDEMIOLOGY
GENETICS
PATHOLOGY

FIGURE 3.

laboration with the Carnegie Institution of Washington and the National Bureau of Standards, on the action of high-energy radiations on various kinds of animal tumors; a comprehensive survey is also under way concerning the health hazards of personnel working in radiological clinics and cyclotron laboratories with a view to reducing these hazards to a minimum; a systematic investigation of the therapeutic action of certain bacterial filtrates; efforts are also continued to discover synthetic chemicals with therapeutic properties; (8) *epidemiological studies* have been pursued for some time with special reference to occupational, racial, medical, social, familial, and environmental background, with a view to determining the extent to which certain factors may be related etiologically to cancer of certain sites; and (9) *clinical research* will be initiated in the near future in collaboration with the new 100-bed tumor clinic at the Marine Hospital in Baltimore, Md.

The National Cancer Institute is proceeding upon the theory that the solution of the cancer problem will not come through the efforts of a single or isolated investigator. During the past two or three decades, no important contributions have been made to our knowledge of cancer except by those who have worked systematically as members of organized research groups. Today our sphere of knowledge has increased to such an extent that no one individual can become skilled in all scientific fields. Hence, cooperative research has become a necessity in cancer research. We need well-organized group efforts. Such organized groups need not kill initiative. Properly organized and staffed, they will release initiative. They will teach the workers the art of working separately together. By this we mean that one can work independently, do his own thinking and yet be sensitive to the ideas and opinions of others, and draw strength from the shared knowledge of the group. Of course, new ideas arise always within an individual. Indeed, every institution and every human achievement was once an idea in the brain of one man. Yet while our ideas and ideals are individual, our techniques in this modern world must be social. This is the method of true science, real liberalism, and genuine democracy.

The responsibilities of the staff of the National Cancer Institute are obvious and the road to achievement is clear!

# THE APPROACHES TO CANCER RESEARCH [1]

By CARL VOEGTLIN, *Chief, National Cancer Institute, National Institute of Health, United States Public Health Service*

It is a readily understandable fact that progress in research resulting in a better control of certain diseases unavoidably leads to a shift in the relative position of these diseases as causes of death.

This has been strikingly reflected in the cancer mortality during the present century. The elimination of typhoid fever by sanitation, the remarkable and continuous decreases in the mortality from tuberculosis, digestive diseases of infants, nutritional and other diseases has greatly increased the average longevity. For this reason and because cancer as a rule requires many years for its development, cancer today is next to "heart disease" as a major cause of death. Cancer has moved from ninth place in 1900 to second place in 1940.

In 1938 there were recorded in the United States 149,000 deaths from cancer. It is estimated that the *ideal* application of all existing knowledge of cancer control would have saved about 25 percent, or 35,000 lives, leaving nearly 115,000 persons doomed to die. Unless prevailing conditions are radically improved, the outlook for the future is even worse, with an estimated total yearly cancer mortality 10 years from now of close to 200,000. Similar statistical data are available from other countries.

What can be done in order to improve this serious situation, apart from the application of recognized control measures? There is only one answer: The acquisition of new knowledge through research. Until recent years cancer research, strange as it may seem, has been a much-neglected field. However, there has fortunately come the realization that the conquest of cancer, as that of other diseases, must come about through scientific investigation.

Cancer is a unique disease. It is not contagious, and there is every reason to believe that it is *not* due to a specific infectious agent. Clinical experience and particularly animal experimentation have clearly shown that cancers are the result of the transformation of normal body cells into malignant cells. The characteristic biological behavior of cancerous tissues must be attributed, therefore, to these permanently altered cells. Cancer cells are endowed with an abnormally high rate of multiplication, as compared with most normal cells. They have the power of invading and destroying normal tissues, thus causing death.

Therefore, the fundamental problem in cancer is to find a satisfactory answer to two questions: (1) The causes for the transformation of normal cells into cancer cells; and (2) the reasons why cancer cells differ from normal cells.

In many respects cancer is not a single disease, but rather a group of diseases, each type of cancer being derived from a different type of normal cell, and each having its peculiar characteristics.

## CAUSATIVE FACTORS

Due largely to experimental research the view is rapidly gaining ground that the

[1] *Paper read before the 8th American Scientific Congress, May 16, 1940, in Washington, D. C., and in modified form at the dedication of the Frank C. Bressler Research Laboratory of the School of Medicine, University of Maryland, May 30, 1940.*

different types of cancer are caused by many and diverse factors. These may be divided into two broad groups: (1) So-called "external" or extrinsic causative factors; and (2) "internal" or intrinsic causative factors. The former act from without, the latter from within the animal body.

The following are examples of external causative factors:

(1) Excessive exposure to X-rays and radioactive material extending over many years as causes of skin cancer and leukemia in certain occupations.

(2) Excessive exposure to strong sunlight (and possibly other atmospheric conditions, i. e., heat, dust) as causes of skin cancer in sailors and farmers.

(3) Certain chemicals used in the manufacture of anilin dyes as causes of cancer of the urinary bladder in factory workers.

(4) Soot and certain coal tars as causes of skin cancer in chimney sweeps and workers handling coal tar.

These so-called "occupational" cancers can be prevented by controlling the respective causative external factors, for instance, by effective protection against radiations, by improvements in manufacturing technique of coal-tar products, etc.

The discovery of some of these external causative factors has opened up a most important field for experimental research and has led to:

(1) The isolation from certain coal tars of a pure and powerful cancer-producing chemical—3,4 benzpyrene.

(2) The chemical synthesis of a very large number of cancer-producing chemicals.

(3) The production of cancers in different organs of animals by means of these chemicals. These cancers have the same characteristics as the cancers in the same tissues of humans.

(4) The possibility of studying the manner whereby these chemicals produce malignant cells.

(5) The opportunity of defining more precisely the rather vague term "chronic irritation" of tissues, which for a long time has been regarded as a likely cancer-producing factor.

I believe that the search for additional external factors has by no means been exhausted. In this connection there is an urgent need for epidemiological and experimental investigations. Careful studies of the precancerous histories of cancer patients with emphasis on environmental conditions and mode of living may well reveal interesting and important information. There are various reasons why such work has been neglected. One of these is the pressure put on the cancer specialist by the never-ending urgent need of diagnosing and treating the large number of patients, leaving little if any time for etiological research. It would seem that progress in this difficult field can only be made by full-time investigators having the necessary training and inquisitive mentality.

But the laboratory investigator also has an opportunity to make his contribution. To mention only two illustrations, he can study on animals the possible relation of atmospheric dust and tobacco smoking to cancer of the lung, or the possible relation of abnormalities in diet to stomach cancer. Cancer of the lung appears to be increasing, and stomach cancer, owing to great difficulties in early diagnosis, accounts for a high percentage of the total cancer mortality. Such work promises to yield important knowledge for the prevention of certain kinds of cancer, just as the discovery of external factors for the above-mentioned occupational cancers has furnished practical preventive measures.

Evidence is also rapidly accumulating indicating that cancer is partly due to *in-*

*ternal factors*. Experiments with animals have clearly shown that abnormalities in the production of certain hormones, especially the sex hormones, are in part responsible for cancer of the breast, the uterus, and the testicle of animals. This promising and complex field is being actively investigated, and much important knowledge will undoubtedly be gained by further systematic work. Suffice it to mention that estrone, one of the hormones of the ovary, is known to produce breast cancer under certain conditions in female as well as male mice.

Now it is evident from the chemical structure of this hormone that it belongs to a very important group of chemicals which occur in the animal body—the steroids. It is of great interest, therefore, to prepare and to test for their cancer-producing properties chemicals belonging to this class, in the hope of discovering compounds which are produced in the body as a result of an abnormal steroid metabolism. The aim is to explain the causation of some of the so-called spontaneous animal and human cancers. This is only one of many striking examples of the part played by chemistry in cancer research.

There are still other internal causative factors. Work with highly inbred strains of mice has clearly shown that heredity plays an important part. We have strains of mice with a very high or very low incidence of breast cancer. Other strains develop at an advanced age a high percentage of lung or liver cancers. There are some indications that hereditary constitution may also play a minor role in human cancer, though it is well to point out that human beings are obviously not inbred.

The principal value of inbred strains of animals is that the hereditary factors can be controlled, thus permitting the study of other causative factors. For instance, it has been shown with eight highly inbred strains of mice that they varied in their susceptibility not only to spontaneous breast and lung tumors, but also to chemically induced subcutaneous and lung tumors. None of them was susceptible or resistant to cancer in general. This is further convincing proof that cancer is a group of diseases and not a single disease.

## BIOLOGY OF CANCER CELLS

This leads us to the most fundamental part of the whole cancer problem—the biology of cancer cells. Now normal as well as cancer cells are by far the most complex physical and chemical organizations known. Our present concept of the physical structure and especially of the chemistry of these cells is very crude indeed. Nevertheless, progress is being made by means of improved technical methods. We know, of course, that the principal parts of cells are the nucleus and the surrounding cytoplasm. For many years investigators have tried to explain the characteristic behavior of cancer cells on the assumption of an abnormality of the minute components of chromosomes, i. e., genes, of the cell nucleus. Some support for this assumption has come from the above-mentioned experiments on hereditary factors. However, until we have more conclusive evidence, it is better to leave this question open and to consider that either nucleus or cytoplasm, or both, undergo a specific change. The possibility that this change may be connected with a specific change of a specific cell protein deserves serious consideration in view of the theoretically unlimited variability in protein structure. This problem, which covers an almost virgin field, is now being attacked.

A vast field has recently been opened up by the discovery of radioactive and non-

radioactive isotopes of the chemical elements, which will serve as an entirely new approach to the study of the metabolism of cancer cells and its relation to the metabolism of the body as a whole. By incorporating these isotopes into the molecular structure of natural organic compounds (amino acids, sugars, etc.) it will be feasible to trace these compounds and their conversion products in their course through the body. By this technique information will be secured concerning the synthesis of the organic constitutents of cancer cells, and normal cells, data which will be of great significance for an understanding of the characteristic growth of cancerous tissues. This shows how modern physics contributes to cancer research.

There are indications that the high fermentation of certain sugars by cancerous tissue plays a role in the destructive action of cancer cells on the surrounding normal cells.

Restriction in the diet of certain amino acids, which are the essential building stones of body proteins and the growth of tissues, causes a striking inhibition of the growth of breast cancer in mice, without apparently interfering with the well-being of the animals. This is of considerable theoretical interest.

Interesting studies are also in progress with tissue enzymes, those important regulators of chemical reactions in cells.

## DIAGNOSTIC TESTS FOR CANCER

A most important line of investigation is the search for tests for the early diagnosis of internal cancers. The curability of these cancers depends on early recognition, before the malignant growth has spread from the original site to more distant parts of the body. The reason why so many lives are lost from cancer of the stomach, liver, and lung—to mention only a few types—is because the early stages of these diseases cannot be recognized except in a small percentage of cases. Tests based on the activity of enzymes in blood serum have either been not specific enough for cancer or they have yielded good results only in the more advanced stage, when a diagnosis can be made by other means. It is very urgent, therefore, to continue the search for more sensitive and specific tests.

## TREATMENT OF CANCER

As first stated, cancer can be cured only if localized. The reason for this is that surgery, X-rays, and radium are measures which can only be applied for the complete destruction of *localized* cancerous growth. While improvements may come from the use of super voltage X-rays and neutrons alone, these powerful radiations have the inherent defect of not only destroying cancer cells but many normal cells as well. In other words, it does not seem at this time that these high-energy radiations could safely be applied in order to cause the destruction not only of the primary, but also of the secondary growths of the more advanced stage of cancer. In this connection we have very recent information showing that the serious hazards incident to exposure of professional personnel to high-energy radiations are by no means fully appreciated. Much work is needed to study the remote action of these radiations in order to safeguard the welfare of physicists and other personnel in cancer clinics. The increasing use of X-rays in industry presents another problem.

A different approach is to discover chemicals with a *specific* action on cancer cells. Much experimental work has been done and is in progress. The proposition is an extremely difficult one, but who could have foreseen only a few years ago the remarkable results now obtained with the sulfanilamide type of drugs in the treatment of many serious diseases? For the

present and until we have a clearer conception of the cancer cell, efforts in this direction will have to be confined to empirical testing of compounds, as was the case in the initial work which led to the discovery of the arsphenamines and sulfanilamide. However, in view of what has been said concerning the diversity of cancers, it is unlikely that a curative chemical agent for all forms of cancer will ever be found. An experimental study of the action of certain bacterial filtrates on cancers of connective-tissue origin is in progress.

## ORGANIZATION OF CANCER RESEARCH

In this brief time it was possible only to sketch some of the approaches of cancer research. Before closing may I be permitted to make some remarks on the ways and means of carrying out such plans. Cancer research is in many respects unique because of the broad scope and the diversity of the problems. The individual scientist, whether biophysicist, chemist, pathologist, and so forth, can never hope to follow his own problem through, because sooner or later he will require the collaboration and criticism of scientists with other training. Cancer research requires team work. Any major cancer-research organization should be closely knit together, without, however, interfering with that most valuable asset—individual imagination and initiative.

The individual worker must be prepared to be interested primarily in the application of his special knowledge to a particular phase of cancer research. In addition he has to acquire, through intimate contact with other specialists, a fairly general view of the cancer problem. Cancer research is a life job and demands unusual courage, imagination, objectivity, and perseverance. Long range and coordinated planning is essential.

Such were the ideas in organizing the National Cancer Institute. In its short existence, this plan has already proved its value.

# INTESTINAL CARCINOMA AND OTHER LESIONS IN MICE FOLLOWING ORAL ADMINISTRATION OF 1,2,5,6-DIBENZANTHRACENE AND 20-METHYLCHOLANTHRENE [1]

By EGON LORENZ, *senior physicist*, and HAROLD L. STEWART, *senior pathologist, National Cancer Institute, United States Public Health Service*

The effect of feeding carcinogenic hydrocarbons to animals has been studied during recent years by a number of investigators (Reinhard and Candee (*1*), Oberling, Sannié, and Guerin (*2*), Cook and collaborators (*3*), Waterman (*4*), Van Prohaska, Brunschwig, and Wilson (*5*), and Magnus (*6*)). Although papillomas and carcinomas of the stomach and neoplasms in the region of the mouth have been reported in a few instances, in most cases no tumors of the gastrointestinal tract were found even after feeding the carcinogen for as long as 10 months. In these experiments the hydrocarbon usually was dissolved in a fatty or oily substance which was mixed with the food, or dropped into the back of the throat with a glass dropper, or fed through a stomach tube. Waterman states that he induced the animals to take the hydrocarbon by licking it off a glass rod.

The present investigations were undertaken for the following reasons: (1) To work out a simple method of oral administration of known quantities of carcinogenic hydrocarbons; (2) to obtain data by absorption spectrum analysis as to the fate of these hydrocarbons in the animal body; and (3) to study the pathological changes produced by long-continued oral administration of these substances.

## MATERIALS AND METHODS

The hydrocarbons 1,2,5,6-dibenzanthracene [2] and 20-methylcholanthrene [3] were administered in an aqueous olive-oil emulsion containing 20 or 10 percent of olive oil and 0.4 or 0.2 mg. per cubic centimeter, respectively, of the hydrocarbon. The minimum melting points of the hydrocarbons were 267° C. corr. and 199.5° C., respectively. The hydrocarbon was dissolved in olive oil heated to approximately 100° C. Distilled water was then added in such proportion as to obtain an emulsion containing 20 or 10 percent of olive oil. Sodium hydroxide was used as an emulsifying agent (8 cc. of a 1 N solution per 100 cc. of olive oil). The mixture was then shaken in a shaking machine for approximately 24 hours. The emulsion was adjusted to a pH of not less than 8.5, with phenol red as indicator. At this pH little creaming out occurred. If the emulsion was stored in an atmosphere of nitrogen, its pH remained unchanged. If stored in an atmosphere of air, the formation of fatty acids by oxidation was counteracted by the addition of sodium hydroxide. The content of hydrocarbon as well as the hydrocarbon itself remained unchanged as shown by repeated absorption spectrum

[1] *This paper was presented in part at the meeting of the American Association for Cancer Research at Richmond, Va., in April 1939, and in part at the meeting of this society in Pittsburgh, Pa., in March 1940. Preliminary reports of some of the results of this work appeared in the annual report of the Surgeon General of the U. S. Public Health Service, 1937 (p. 63) and 1938 (p. 82).*

[2] *Hereafter referred to as dibenzanthracene.*

[3] *Hereafter referred to as methylcholanthrene.*

analyses. For such determinations, 1 cc. of the emulsion was extracted with ether after breaking the emulsion with hydrochloric acid. Then the ultraviolet absorption spectrum of the extract was compared with standard spectra of the hydrocarbon (7). The emulsion was administered to the animals in wide-mouthed bottles of 100-cc. capacity, closed by a rubber stopper, into which a glass tube 8 mm. in diameter and 10 cm. long was inserted. It was given in place of drinking water.[4] Records of the weight of the animals and of the quantity of emulsion consumed were kept. The average daily intake of emulsion per mouse was computed, as well as the total intake of hydrocarbon for the last surviving mouse. By shaking the feeding bottles at least once a day and by using the emulsion left in the bottles, the average content of the hydrocarbon in the emulsion could be maintained. Losses from spilling were estimated to be less than 5 percent. Table 1 gives the data on the emulsions used and their consumption.

A relatively small number of animals died during the course of the experiments. Animals in extremis were killed. Animals were also killed at intervals for weight records of organs, for pathological studies, and to obtain spectrographic data on the fate of the hydrocarbons. The mice were killed with ether or by cervical dislocation. The autopsy was performed immediately. When the organs were weighed they were removed from the body, cleaned of all adhesions and fat, and weighed on a torsion balance with a range of 500 mg. which could be extended to 1,500 mg. by counterweights. The organs and structures of the abdominal, thoracic, cranial, oral, and nasopharyngeal cavities were examined, together with the structures and organs of the face, neck, superficial and deep lymph nodes, cutaneous, muscular, peripheral nervous, and osseous tissues, and bone marrow from vertebrae and occasionally from ribs, scapulae, pelvis, and femur. When the animal died it was not always possible to carry out the autopsy immediately after death, but in the study of the tissue sections ample allowance was made for the possible com-

[4] *The tip of the glass tube was narrowed to an opening, a few millimeters in diameter. These bottles were placed perpendicularly through the lid (screen wire $\frac{3}{8}$-inch mesh) of the wooden box (12 by 8 by 6¼ inches) in which the mice were kept (10 mice to a box). Sawdust was used for bedding and was changed twice weekly.*

TABLE 1.—*Consumption of emulsion by mice of strain A and strain A backcrosses*

| Experimental group | Age of mice | Strain | Number | Sex | Duration of experiment | Emulsions | | | Quantity invested by any one mouse | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Olive oil | Hydrocarbon | Hydrocarbon per cc. | Total average of— | | |
| | | | | | | | | | Emulsion per day | Hydrocarbon per day | Maximum hydrocarbon |
| | *Months* | | | | *Months* | *Percent* | | *Mg.* | *Cc.* | *Mg.* | *Mg.* |
| 1 | 3 | A backcross | 9 | M | 9 | 20 | D. B. A.[1] | 0.4 | 1.8 | 0.72 | 174 |
| 2 | 1½ | do | 10 | M | 13 | 10 | do | .2 | 2.5 | .5 | 182 |
| | | | 10 | M | 13 | 10 | | | 4.5 | | |
| 3 (A)[2] | 4 | A | 10 | M | 8 | 20 | D. B. A.[1] | .4 | 2.0 | .8 | 160 |
| 3 (B) | 4 | A | 10 | M | 10 | 20 | do | .4 | 1.6 | .64 | 156 |
| 3 (C) | 4 | A | 10 | F | 9 | 20 | do | .4 | 2.1 | .84 | 192 |
| 4 | 4 | A backcross | 10 | M | 10 | 20 | M. C. A.[3] | .4 | 2.2 | .88 | 275 |
| 5 (A)[2] | 4 | do | 10 | M | 8 | 20 | D. B. A.[1] | .4 | 2.2 | .88 | 177 |
| 5 (B) | 4 | do | 10 | M | 8 | 20 | do | .4 | 2.1 | .84 | 180 |
| 6 | 4 | do | 10 | M | 7 | 20 | do | .4 | 2.2 | .88 | 181 |

[1] Dibenzanthracene. [2] Letters in parenthesis indicate subgroups. [3] Methylcholanthrene.

plicating factor of post mortem autolysis. Specimens of all the various organs and tissues examined were usually fixed in Zenker's fluid; occasionally other fixatives were used, as formaldehyde (U. S. P. 1:10), Tellyesniczky's fluid, and Bouin's fluid. Some tissues were frozen and stained for fat; the rest were embedded in paraffin, cut, and stained with haematoxylin and eosin, eosin methylene blue, phosphotungstic acid haematoxylin, van Gieson's and Mallory's connective tissue stains, Foot's silver stain for reticulum, and Mallory's potassium ferrocyanide stain. The pituitary was blocked in paraffin, cut in serial sections, and stained with Mallory's anilin blue stain.

The organs and tissues for spectrographic analysis were minced with scissors, and the wet tissue was extracted by shaking with ether for several hours. It was shown that three consecutive extractions were sufficient to obtain the hydrocarbon quantitatively in solution in ether. The ether was evaporated to 5- or 10-cc. quantities and the extract subjected to absorption spectrum analysis. The ether extracts of most tissues were sufficiently free from absorbing substances which might interfere with the spectrographic determination. Further extraction was necessary in some instances since absorption by extracted lipoids and pigments blotted out the characteristic absorption bands of the hydrocarbon on the photographic plate. The ether extract was evaporated to dryness, and the dry material was dissolved in methyl alcohol. By this procedure some of the interfering material failed to go into solution. The methyl alcohol solution was then subjected to absorption spectrum analysis. Additional methods of purification which were also used are described elsewhere (7). The minimum amount detectable by this method is dependent upon the amount of interfering material present. It is usually between $5\times10^{-4}$ and $1\times10^{-3}$ mg. of the hydrocarbon per organ or per cubic centimeter of body fluid.

Inbred strain A mice and strain A backcross mice weighing between 20 and 30 gm. were used. The strain A backcross mice were obtained by mating the $F_1$ hybrids of C57 black and A mice to the parent A stock. All the mice were purchased from the Roscoe B. Jackson Memorial Laboratory at Bar Harbor, Maine. They were fed Purina dog chow, which contained, according to the manufacturer, 20 percent protein, 3 percent fat, 56 percent carbohydrate, 6 percent ash, 15 percent water, and vitamins A and G, pressed in convenient checkers. No measurements of the food intake were taken. As stated, the source of the fluid intake for the experimental mice was the emulsions. Water was allowed only when the laboratory was moved, and for therapeutic purposes whenever the animals developed ascites or lost weight rapidly (fig. 1). These manifestations will be discussed later.

### GROUP 1

During June 1937, nine male strain A backcross mice approximately 3 months old were given orally an aqueous olive-oil emulsion containing 20-percent olive oil and 0.4 mg. dibenzanthracene. After 3 months' treatment most of the animals appeared ill and were given water for approximately 1 month. The last mouse was killed after 9 months' treatment; the amount of dibenzanthracene ingested by this mouse was 174 mg., and the average daily intake of emulsion was 1.8 cc. containing 0.72 mg. of dibenzanthracene (table 1).

### GROUP 2

To investigate the effects of smaller doses of the hydrocarbon, the dibenzanthracene content in the emulsion was reduced to 0.2

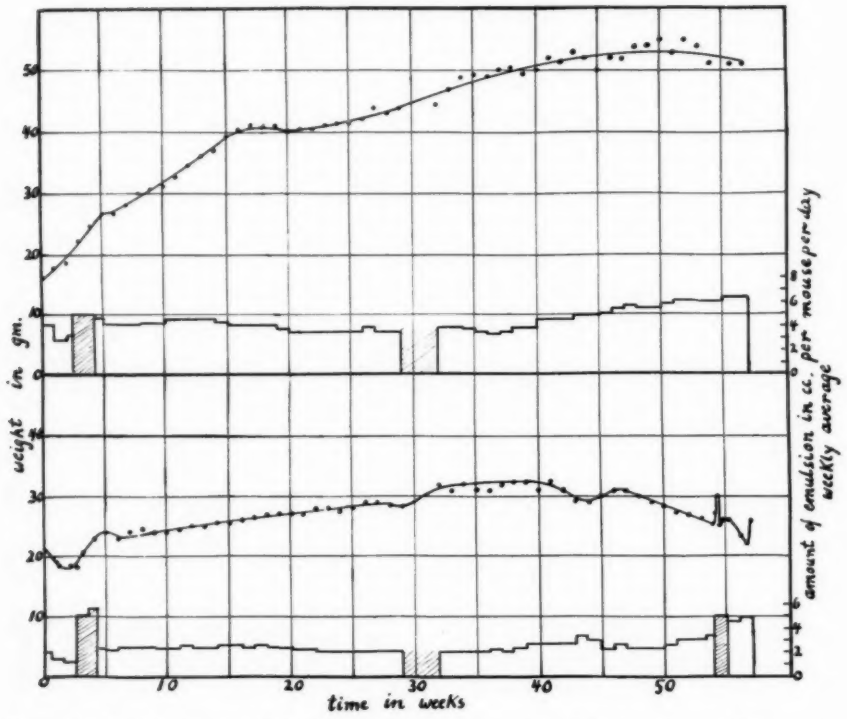



FIGURE 1.—Weight curves and daily average consumption of emulsion per mouse. Upper curve: Aqueous olive-oil emulsion (10-percent olive oil). Lower curve: Aqueous olive-oil emulsion (10-percent olive oil) containing 0.2 mg. of dibenzanthracene per cubic centimeter of emulsion. Shaded areas represent periods during which drinking water was given.

mg. per cubic centimeter and that of olive oil to 10 percent. Strain A male backcross mice 1½ months old were used (November 1937). Ten mice were given the experimental emulsion; 10 control mice were given an aqueous olive-oil emulsion containing 10 percent of olive oil; 5 control mice were given drinking water. These 5 mice were kept on sawdust bedding (approximately 100 gm.) to which 20 cc. of an aqueous olive-oil emulsion containing 8 mg. of dibenzanthracene was added and mixed. The bedding was changed weekly. The object was to determine whether the mice would inhale dust particles containing sufficient hydrocarbon to induce lung tumors.

The last experimental mouse survived for 13 months; the amount of dibenzanthracene ingested by this mouse was 182 mg., and the average daily intake of emulsion 2.5 cc. containing 0.5 mg. of the hydrocarbon. Eight of the control mice ingesting olive-oil emulsion were killed 13 months after the beginning of the experiment, one died, and one was killed previously. Of the five mice living on sawdust mixed with the dibenzanthracene emulsion, one was killed after 8 months, a second after 10 months, and three were

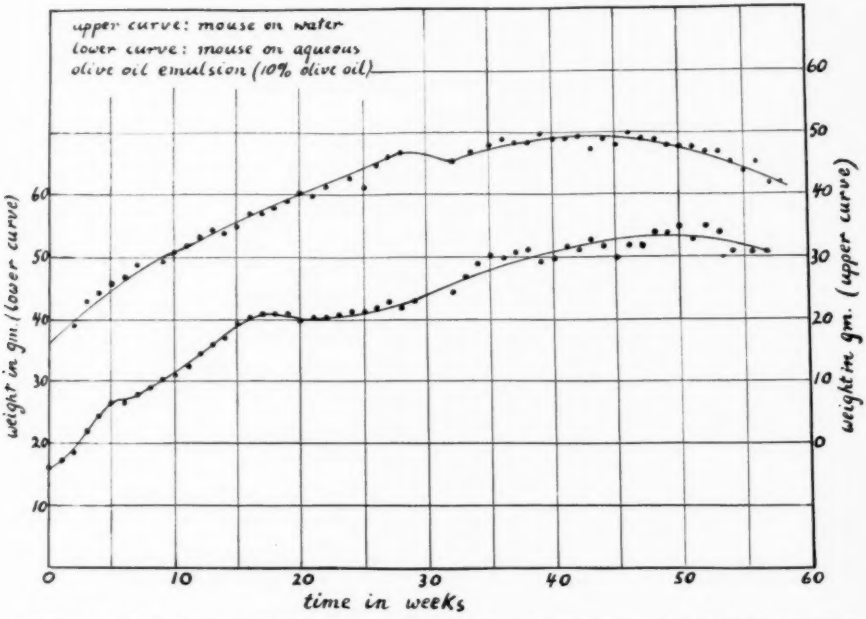



FIGURE 2.—Weight curves of control mice ingesting aqueous olive-oil emulsion and drinking water.

killed 13 months after the beginning of the experiment. The control mice given olive-oil emulsion ingested an average of 4.5 cc. daily, and those receiving drinking water between 4 and 6 cc. daily. Figure 1 gives the weight curve and the curve of the daily intake of emulsion (weekly averages) for a single animal each, of the experimental and control series. Figure 2 gives the weight curves of two control mice, one ingesting olive-oil emulsion and the other drinking water.

GROUP 3

To study the effects of feeding dibenzanthracene to a different strain of mice, male and female strain A mice aged 4 months were used (April 1939); 2 subgroups of 10 male mice each (A and B), and 1 subgroup of 10 female mice (C), were given an aqueous olive-oil emulsion containing 20 percent olive oil and 0.4 mg. per cubic centimeter of dibenzanthracene; 5 male and 5 female mice served as controls and were given drinking water. Table 2 gives the data on the weights of the animals and organs.

The last mouse of subgroup A survived for 8 months. It had ingested a total of 160 mg. of dibenzanthracene. The last mouse of subgroup B ingested in 10 months a total of 162 mg., and the last mouse in subgroup C ingested in 8 months a total of 192 mg. The average daily intake of emulsion in subgroup A was 2.0 cc. containing 0.8 mg. of the hydrocarbon, that of subgroup B, 1.6 cc. containing 0.64 mg., and that of subgroup C, 2.0 cc. containing 0.8 mg. (table 1).

The first carcinomas of the small intestine appeared in these mice as follows: Subgroup A at 7 months after ingesting 125 mg. of dibenzanthracene; subgroup

TABLE 2.—*Weight of animals and various organs of strain A mice ingesting dibenzanthracene emulsion*

GROUP 3

| Duration of exposure (months) | Body weight | Sex | Anterior prostate | Testes and epididymis | Seminal vesicles, coagulating glands, vas deferens, posterior prostate, and bladder | Uterus and ovaries | Kidneys | Liver | Spleen |
|---|---|---|---|---|---|---|---|---|---|
| | *Grams* | | *Gram* | *Gram* | *Gram* | *Gram* | *Gram* | *Gram* | *Gram* |
| 1 | 21 | F | | | | | 0.307 | 1.594 | 0.120 |
| 2 | 21 | M | 0.032 | 0.189 | 0.194 | | .264 | 1.264 | .068 |
| | 21 | F | | | | 0.028 | .265 | 1.203 | .073 |
| 3 | 19 | M | .031 | .123 | .080 | | .282 | 1.184 | .033 |
| 6 | 17 | F | | | | .035 | .335 | 1.444 | .120 |
| 7 | 19 | M | .043 | .210 | .112 | | .296 | 1.282 | .098 |
| | 14 | M | .019 | .161 | .080 | | .277 | .834 | .066 |
| CONTROLS | | | | | | | | | |
| 1 | 23 | F | | | | 0.150 | 0.282 | 1.220 | 0.094 |
| 6 | 40 | F | | | | .058 | .459 | 1.428 | .096 |
| | 28 | F | | | | | .440 | 1.372 | .112 |
| 11 | 35 | F | | | | .074 | .492 | 1.625 | .166 |
| | 33 | F | | | | .070 | .410 | 1.486 | .080 |
| | 39 | M | 0.120 | 0.342 | 0.366 | | .522 | 1.906 | .166 |
| 12 | 35 | M | .094 | .320 | .594 | | .504 | 1.734 | .082 |
| | 36 | M | .158 | .300 | .550 | | .512 | 1.880 | .175 |

B at 8½ months after ingesting 130 mg.; subgroup C at 7¾ months after ingesting 147 mg. One mouse with intestinal carcinoma was found in subgroup A, five in subgroup B, and one in subgroup C (table 3). Most mice of subgroup A and C had either died or were killed prior to the time of appearance of the first tumor.

GROUP 4

In April 1939, 10 strain A backcross male mice, 4 months old, were given an aqueous olive-oil emulsion containing 20 percent olive oil and 0.4 mg. of methylcholanthrene (melting point 179.5° C., corr.) per cubic centimeter. The last mouse of this group survived for 10 months; it had ingested a total of 275 mg. of methylcholanthrene. The average daily intake of emulsion was 2.2 cc. containing 0.88 mg. of this hydrocarbon (table 1).

The first intestinal tumor (table 3) was observed in a mouse at 7½ months after ingesting 174 mg. of methylcholanthrene.

TABLE 3.—*Induced intestinal tumors in strain A and strain A backcross mice*

| Strain of mice | Number | Sex | Hydrocarbon administered | Hydrocarbon ingested per mouse per day (average) | Total hydrocarbon ingested per mouse until first tumor appeared (average) | Time of appearance of first tumor | Mice with intestinal tumors | Mice with two intestinal tumors | Metastasis | Positive transplantation |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Mg.* | *Mg.* | *Months* | *Number* | *Number* | | |
| A | 10 | Male | Dibenzanthracene | 0.8 | 125 | 7 | 1 | | | |
| A | 10 | do | do | .64 | 113 | 8½ | 5 | 2 | | |
| A | 10 | Female | do | .84 | 145 | 7¾ | 1 | | | |
| A backcross | 10 | Male | Methylcholanthrene | .88 | 174 | 7½ | 3 | 1 | 1 | 1 |

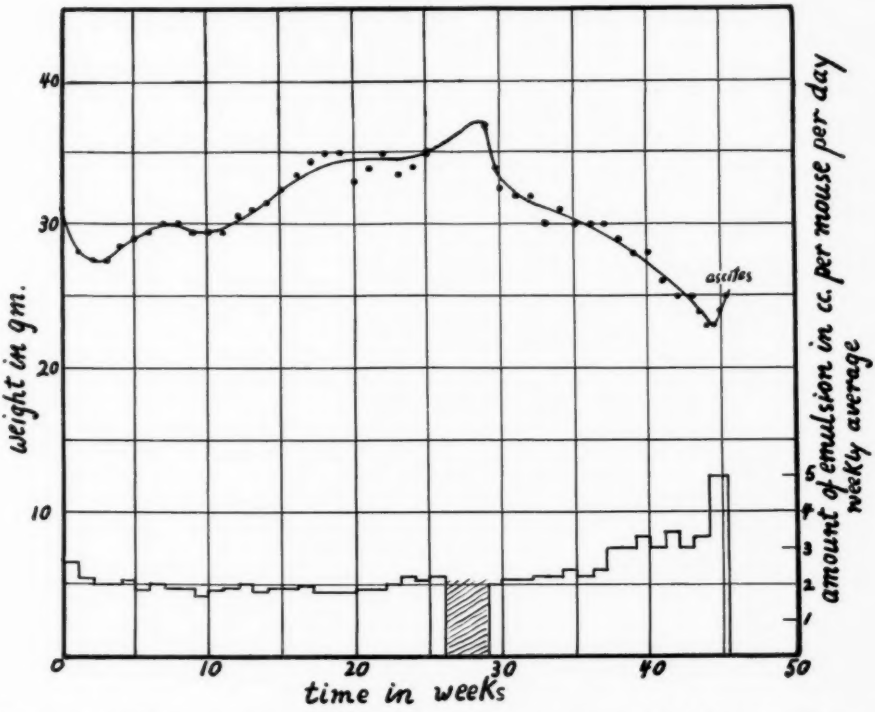



FIGURE 3.—Weight curve and daily average consumption of aqueous methylcholanthrene-olive oil emulsion (20 percent olive oil, 0.4 mg. of methylcholanthrene per cubic centimeter of emulsion). Shaded area represents period during which drinking water was given.

Two of the four mice surviving this animal also developed intestinal carcinoma.

The mice did not become ill until near the end of the experiment when two mice developed ascites. The mice were given the methylcholanthrene emulsion for the entire duration of the experiment except for 3 weeks when the laboratory was moved, during which time they were given water. Figure 3 shows the weight curve and the average daily intake of emulsion of an experimental mouse of this group. Table 4 compares the weights of some experimental animals and their organs with that of control animals and their organs.

GROUP 5

Twenty strain A backcross mice 4 months old, divided into 2 subgroups (A and B) were given an aqueous olive-oil emulsion containing 20 percent of olive oil and 0.4 mg. of dibenzanthracene per cubic centimeter (April 1939). In addition they were injected subcutaneously with 0.1 mg. of testosterone proprionate in 0.1 cc. of sesame oil [5] twice a week for 9 weeks, and once a week thereafter. The last surviving mouse (subgroup B) received 42 injections totaling 4.2 mg. of

[5] *We are indebted to Dr. Gregory Stragnell of the Schering Corporation, Bloomfield, N. J., for supplying the testosterone proprionate and the sesame oil which was used in preparing solutions.*

testosterone proprionate. Testosterone proprionate was given because the male mice ingesting dibenzanthracene developed severe atrophic changes of the genital organs. The possibility was considered that the period of treatment could be lengthened if this atrophy could be prevented or retarded.

TABLE 4.—*Weight of animals and various organs of strain A backcross mice ingesting methylcholanthrene emulsion (group 4 and controls)*

GROUP 4

| Duration of exposure (months) | Body weight | Anterior prostate | Testes and epididymis | Seminal vesicles, coagulating glands, vas deferens, posterior prostate, and bladder | Kidneys | Liver | Spleen |
|---|---|---|---|---|---|---|---|
| | *Grams* | *Gram* | *Gram* | *Gram* | *Gram* | *Grams* | *Gram* |
| 1 | 25 | 0.062 | 6.269 | 0.250 | 0.346 | 1.518 | 0.253 |
| 3 | 25 | .056 | .272 | .296 | .339 | 1.218 | .217 |
| 7 | 20 | .100 | .326 | .328 | .459 | 1.374 | .158 |

CONTROLS

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 3 | 37 | 0.082 | 0.343 | 0.410 | 0.520 | 1.524 | 0.272 |
| 7 | 34 | .102 | .310 | .318 | .596 | 1.613 | .160 |
| 8 | 33 | .080 | .260 | .273 | .474 | 1.768 | .236 |

The average daily intake of emulsion per mouse was 2.2 cc. containing 0.88 mg. of dibenzanthracene in subgroup A, and 2.1 cc. containing 0.84 mg. in subgroup B. The last surviving mouse of subgroup A which died after 8 months of treatment had ingested 177 mg. of dibenzanthracene, and the last surviving mouse of subgroup B, killed after 8 months of treatment, had ingested 180 mg. (table 1).

GROUP 6

The mice of group 6 were also male strain A backcross, aged 4 months (April 1939). They were given the same emulsion as the mice in group 5, and in addition were injected subcutaneously with 0.1 cc. of sesame oil twice a week for 9 weeks, and once a week thereafter. The last surviving mouse received 39 injections totaling 3.8 cc. of sesame oil.

The average daily intake of emulsion per mouse was 2.2 cc. containing 0.88 mg. of dibenzanthracene. The last surviving mouse, which died after 7 months of treatment, had ingested 181 mg.

Three subgroups of control mice for groups 5 and 6, five in each group, were given drinking water. The first group received subcutaneous injections of 0.1 mg. of testosterone proprionate in 0.1 cc. of sesame oil twice weekly for 9 weeks, and weekly thereafter for a total of 42 injections. The second group received subcutaneous injections of 0.1 cc. of sesame oil twice weekly for 9 weeks, and weekly thereafter, totaling 42 injections. The third group received no treatment.

It was difficult to keep the experimental mice of these two groups alive. They developed ascites frequently and had to be given drinking water. In almost all the animals autopsied oil pockets were observed, which were frequently infected. The results obtained from this experiment are considered of little value.

All experimental mice ingesting hydrocarbons developed multiple induced tumors of the lung. In order to rule out the possibility of these induced lung tumors resulting from aspiration of the hydrocarbon, the following experiment was performed: Seven male ABC mice, aged 3 months, were administered a charcoal suspension in water for 4 months beginning in August 1939. The aqueous charcoal suspension containing 1 mg. of charcoal per cubic centimeter was prepared by adding charcoal to distilled water and shaking briefly in a separatory funnel. The larger particles were allowed to settle out. The supernatant liquid was filtered, and after drying the residue of charcoal

particles, a suspension of 1 mg. of this charcoal in water was prepared to which a wetting agent, 0.14 percent of Aerosol OT [6] (the dioctyl ester of sodium sulfosuccinic acid), was added to retard the settling out. As the charcoal still settled out if the suspension stood overnight, it was administered only during the daytime and shaken every 2 hours. No fluids were administered at night. The lungs of these mice were fixed by intratracheal injection of Zenker's fluid, and after blocking them in paraffin a number of sections were cut at different levels. Histologic sections were examined for the presence of charcoal particles to determine whether aspiration of the drinking mixture occurred.

## RESULTS

### ABSORPTION SPECTRUM ANALYSES

Absorption spectrum analysis of tissues and body-fluid extracts revealed the presence of dibenzanthracene, in unchanged form in the gastrointestinal tract down to the level of the large intestine. In the large intestine the hydrocarbon was not found within the limit of sensitivity of the method, which was $1\times10^{-4}$ mg. of the hydrocarbon for this viscus. However, a new band spectrum was observed in extracts of the large intestine, similar to the spectrum of dibenzanthracene but with some bands broadened and shifted toward longer wave lengths. This suggested a conversion product of dibenzanthracene. This new band spectrum was also observed in the ether extract of feces. Dibenzanthracene was found in the feces only in traces; 135 gm. of dried feces contained 0.6 mg. of nonconverted dibenzanthracene. As these feces were collected for months from the sawdust on which the mice lived, the possibility was considered that the nonconverted hydrocarbon resulted from spilled emulsion. It was assumed, therefore, that no dibenzanthracene as such remained in the large intestine.

Extracts of blood, ascitic fluid, fat, liver, and lungs failed to show the presence of the hydrocarbon except in the case of two of six pairs of lungs in which small amounts of the hydrocarbon were found. Inasmuch as such small quantities as $1\times10^{-3}$ mg. per organ or per cubic centimeter of fluid or per gram of fat could have been detected, it must be concluded that the concentration of the hydrocarbon in the body tissues and fluids outside the gastrointestinal tract and except for the two pairs of lungs mentioned above was less than $1\times10^{-3}$ mg. per organ or per cubic centimeter of fluid or per gram of fat. Contamination of the two specimens of lungs might have occurred accidentally. Inhalation of sawdust particles containing dibenzanthracene could not have been responsible for these findings since the control mice of group 2 which lived on sawdust mixed with the dibenzanthracene emulsion, did not develop induced pulmonary tumors. Some of these animals survived for 12 months. Aspiration of the emulsion during the act of drinking also could not have been the cause of the induced lung tumors in the experimental mice, as mice which were given drinking water containing charcoal for 5 months did not show charcoal particles in the lung.

No spectrographic data on the fate of methylcholanthrene are available as yet.

## CLINICAL OBSERVATIONS

Figure 1 gives the weight curve and the curve of the daily intake of emulsion (weekly averages) for a single experimental and a control mouse of group 2. The curves are representative of all the mice used in this experiment. The curve for the mouse on the aqueous olive-oil

[6] *Manufactured by the American Cyanamid Co.*

emulsion containing 0.2 mg. of dibenzanthracene per cubic centimeter shows that the weight of the animal decreased rapidly at the beginning of the experiment. This may be owing in part to the fact that since the animal disliked the emulsion containing the hydrocarbon, the intake of fluids was diminished. The mouse was then given water for approximately 10 days, and the fluid-intake and weight curve rose rapidly. When it was again returned to the emulsion, the intake of fluid decreased once more but reached a level of approximately 2.5 cubic centimeters per day which was apparently sufficient to maintain life. For the succeeding 8 months the intake of fluids was fairly constant, and weight increased slowly but consistently. For 3 weeks during this time when the laboratory was moved, the mouse was given water again; it responded with a somewhat more rapid increase in weight. After 8 months the weight curve began to drop. After 12 months there was a sudden rapid increase in weight coincident with the development of ascites, and water was substituted for the emulsion. The weight as well as water intake rose rapidly for a few days, but the weight dropped promptly to normal as the ascites disappeared. When returned to the dibenzanthracene regime there was a recurrence of ascites, and the animal was killed. A comparison of the weight curve of the mouse given the emulsion containing dibenzanthracene with that of the mouse on plain olive-oil emulsion shows that the latter curve rose more steeply and continued to rise almost to the end of the experiment. The fact that the weight of the control mouse on plain olive-oil emulsion was at all times considerably greater during the experiment than that of the experimental animal ingesting dibenzanthracene emulsion, demonstrated that the lower weight of the experimental animal was not due to the olive-oil content of the emulsion containing the hydrocarbon.

Figure 2 gives the weight curve of a mouse of group 2 ingesting plain olive-oil emulsion and of a control mouse ingesting drinking water. Both curves are similar, indicating that the high fat diet of the mouse ingesting the olive-oil emulsion was not detrimental to the health of the animal. This observation was corroborated by comparative pathological studies of these animals.

Figure 3 gives the weight and fluid-intake curve of a mouse of group 4 ingesting an aqueous methylcholanthrene emulsion containing 20 percent of olive oil and 0.4 mg. of methylcholanthrene per cubic centimeter. There was a slow, steady increase in weight up to approximately 6 months, followed by a gradual decline in weight, which became more abrupt toward the end of the experiment. The terminal increase in weight was due to ascites.

No weight and fluid-intake curves were given for the mice ingesting 0.4 mg. of dibenzanthracene because of marked fluctuations in weight. It was difficult to keep these mice alive, especially those of group 3. The main difficulty was not so much the development of ascites, which occurred occasionally, as the frequent alarming drop in weight which occurred when the mice were given the emulsion for longer than approximately 1 month. Some of the mice of subgroup A which were under treatment for 8 months received water intermittently for a total of 2 months of this period.

Clinically, many animals became emaciated, those with dark hair became gray and prematurely old, and the skin of the paws and mucous membranes pale. The animals in group 2 ingesting dibenzanthracene and those in group 4 ingesting methylcholanthrene were active and ate well during the experiment, while this was

not the case in the other groups owing probably to the higher content of dibenzanthracene in the emulsion. The changes of premature aging went hand in hand with atrophy of the organs and emaciation and was pronounced in mice 10 to 12 months old. Occasionally mice lost weight rapidly within a few days probably owing to insufficient ingestion of fluids in some instances. Usually these animals recuperated rapidly when drinking water was substituted for the emulsions. When weight loss was associated with intestinal neoplasm, it continued regardless of treatment and the animals had to be killed. In contrast a number of mice made rapid increase in weight because of an accumulation of fluid in the subcutaneous tissue and body cavities, more especially in the peritoneal cavity. The latter manifestation, characterized by marked distention of the abdomen, was detectable by physical examination. The increase in weight often amounted to several grams (maximum 6 gm.) within a few days. By depriving the animals of the emulsion and substituting small amounts of drinking water, the edema usually disappeared and the animals were then returned to the emulsion regime. However, if at this stage an unlimited amount of water was allowed, the anasarca increased and the animal died.

## PATHOLOGICAL OBSERVATIONS

### ANASARCA

As mentioned above many mice developed anasarca. The first case in mice ingesting dibenzanthracene emulsion appeared after 3 weeks, and mice autopsied thereafter were apt to show some signs of this condition. Animals ingesting methylcholanthrene on the other hand did not develop anasarca until after 8 months. Ascites was particularly prominent. The character, color, and amount of the ascitic fluid varied. Usually 1 or 2 cc. could be measured at autopsy, but in one case 4 cc. were recovered. In most cases the fluid was clear but frequently contained small oil droplets upon microscopic examination. In a number of cases, however, the ascitic fluid was thick, white, and opaque and had a chylous appearance. Ascitic fluids of intermediate composition were also observed. In a few cases of chylous ascites there was associated bilateral chylous effusion into the pleural cavities. One mouse developed hemorrhagic ascites, and another hemorrhagic pleural effusion. A number of specimens of ascitic fluid from mice on the dibenzanthracene regime were extracted with ether and subjected to absorption spectrum analysis. This hydrocarbon was not found spectroscopically in these fluids within the limits of the sensitivity of the method, which in this case was $5 \times 10^{-4}$ mg. of dibenzanthracene per cubic centimeter. In two cases the chylous ascitic effusion was collected and injected subcutaneously into other mice. Three mice injected with one sample all died within 24 hours with extensive necrosis at the site of injection. The animals injected with the other sample are alive, 3 months later, and show no significant change.

### LUNGS

The oral administration of both methylcholanthrene and dibenzanthracene elicited the development of multiple tumors of the lungs. Except for the specimens described later these tumors presented the gross and microscopic characteristics of tumors induced in the lungs of mice by injection or painting with the carcinogenic hydrocarbons.

All the control mice ingesting water or plain olive-oil emulsions, as well as the mice living on sawdust bedding to which the dibenzanthracene emulsion was added, showed no increase of lung tumors over

the normal spontaneous incidence. Histologic study of the lungs of control mice drinking charcoal suspension revealed the total absence of charcoal particles.

In the experimental animals pulmonary growths were observed microscopically after 1½ months of treatment and grossly at autopsy after 5 months. The grossly visible tumors increased in size and number with the progress of the experiment. The largest single growth observed occupied an entire upper lobe of the lung. The greatest number of tumors counted on the pleural surfaces of the lungs of any one animal was 100 to 110. This animal had ingested methylcholanthrene for 8 months. In the histologic sections of several lungs with multiple tumors and in one antedating the appearance of tumor there was a pronounced hyperplasia of the septal cells. There was an infiltration of myeloid cells in the septa of the lungs of a mouse ingesting dibenzanthracene, and in two others receiving in addition injections of testosterone proprionate. Bronchopneumonia was present in 9 mice in the various groups.

One lung specimen showed a tumor, the characteristics of which differed from those of the ordinary type. The lung contained large tumor masses varying considerably in different sections. In many places the tumor showed a papillary structure. The cores of the papillae consisted of an extremely thin bundle of connective tissue covered by one or more layers of large columnar or cuboidal tumor cells. These cells possessed single, large, more or less rounded, darkly staining nuclei. Sometimes the cores of the papillae appeared larger, and frequently in these instances they suggested invasion by tumor cells. Frequently associated with this type of growth were areas in which the tumor cells formed small, rounded acini, lined by a single or multiple layer of tumor cells similar to those covering the papillae. In still other areas, the tumor grew in the form of rather solid, irregular sheets composed of extremely large cells with large, darkly staining nuclei which frequently contained hyaline, slightly acidophilic, intranuclear inclusion bodies. Around the periphery of several areas of this type the alveolar walls of noninvaded lung tissue were seen to be partially lined by large, irregular mononuclear cells closely resembling definite tumor cells. In many instances also, the alveolar spaces were partly filled with clumps of similar cells. There were several areas of necrosis and about one of these were found thin, long, and sometimes curving bundles of spindle cells with elongated darkly staining nuclei and relatively smooth cytoplasm. The nuclear characteristics of these spindle cells differed markedly from the obviously epithelial tumor cells noted before and did not appear to represent compressed or elongated forms of epithelial tumor cells. There were several areas of inflammation characterized by clumps of degenerated polymorphonuclear leukocytes, small numbers of red blood cells, and fibrin, and in these areas the alveolar walls appeared to be partly destroyed. The bronchi sometimes contained a small amount of purulent exudate; and in at least one instance where a major bronchus was cut longitudinally, it was compressed almost to obliteration of the lumen by the tumor.

### LIVER

The livers of two-thirds of the animals under treatment showed no visible change grossly or microscopically. In groups 2, 3, and 6 there were a few animals with livers which appeared smaller than normal. In groups 1, 2, and 3 there was an occasional mouse with increase in the pigment of the liver which appeared to be deposited chiefly in Kupffer's cells. Six

mice in groups 1, 2, 3, and 6 showed multiple small focal areas of bile duct proliferation (pl. 1, *A*). The animals presenting these lesions had been under treatment for 5 months or more. Only one case of cirrhosis was observed, and this occurred in a mouse in group 3. Grossly, the liver of this animal showed a finely granular surface and accentuation of the liver markings. Microscopically, there was an increase of fibrous tissue in the portal areas in association with proliferation of small bile ducts and an infiltration of a few inflammatory cells, without, however, any very extensive degeneration or necrosis of the hepatic cells. In groups 3 and 6 the livers of a few animals showed multiple small focal necroses. There was an infiltration of cells whose appearance and arrangement suggested leukosis in the livers of five mice in group 5 and in two mice in group 6. In five cases the infiltrating cells appeared to be of the myeloid type, but in two cases their identification could not be made with certainty. An interesting negative finding in these experiments was the absence of any marked fatty vacuolization of the liver cells despite the fact that the mice were ingesting a high fat diet for long periods of time. No significant changes were found in the livers of the mice ingesting methylcholanthrene.

### KIDNEYS

The kidneys were relatively slightly affected by the treatment. Animals in group 2 and occasionally those in other groups showed kidneys somewhat reduced in size but with little change microscopically. In a few animals in the different groups there was slight flattening of the lining epithelium of portions of the tubule, but only one animal had marked nephritis.

### SPLEEN

Atrophy of the spleen occurred in the mice of groups 1, 2, 3, 5, and 6. The development and degree of splenic atrophy among the animals of the same and different groups varied somewhat, although in all animals receiving the dibenzanthracene emulsions for 5 months or more this lesion was pronounced (table 3). In several animals autopsied after ½ to 3 months of treatment the spleen showed slight to moderate atrophy. The most marked atrophy appeared to occur in the mice in group 5. Of the animals given methylcholanthrene five presented a normal spleen at autopsy, although they had ingested the emulsion from 1½ to 7½ months; in three other mice fed 8½ to 10 months there was only slight atrophy.

Microscopically, both the follicles and the pulp were involved in the early stages, but as a general rule the pulp cells showed the more pronounced depletion. In the late stages of feeding, the spleen was reduced to a small organ composed of closely approximated trabeculae, dilated sinusoids, and an accumulation of a brown pigment within large phagocytic cells tending to be distributed along the trabeculae (pl. 1, *B*). There was only an occasional isolated phagocytic cell containing iron pigment. When the lesion was far advanced, the remaining few cells between the trabeculae consisted chiefly of lymphocytes, mononuclear leukocytes, a few spindle cells, and an occasional myeloid cell. No follicles could be recognized. In several cases of leukosis the pulp was infiltrated with myeloid cells or contained other cells which resembled hyperplastic reticulum cells. In a few specimens there were large numbers of immature red blood cells and multiple thrombi in the splenic vessels. In one mouse in group 4 the spleen showed chronic capsulitis.

### OTHER LYMPHOID TISSUE

In the animals which were given dibenzanthracene and which showed atrophic changes in the spleen, there was corre-

sponding atrophy of the lymphoid tissue throughout the body affecting the lymph nodes generally, the lymph follicles in the wall of the intestine, and to a certain extent the thymus. The lymph nodes in the animals fed the methylcholanthrene emulsion showed practically no atrophy, and in some cases hyperplasia. The mesenteric lymph nodes from the mice with carcinoma of the small intestine showed inflammatory hyperplasia and in one case metastatic carcinoma as described below.

### BONE MARROW

Specimens of the bone marrow were obtained in practically all cases. Most of these were from the vertebrae, but occasionally other sites were selected, such as the scapula and the long bones. In the mice given methylcholanthrene no significant changes were observed in the vertebral bone marrow. On the other hand many mice fed dibenzanthracene for 5 months or longer showed a variable degree of marrow-cell depletion, affecting all types of cells and most marked in the long and flat bones of animals which had received the emulsion for the longest periods (pl. 1, *C*). Coincident with the depletion of the marrow cells the marrow cavities became occupied by fat and congested vessels.

### GENITAL ORGANS

The animals in groups 1, 2, 3, and 6 ingesting 1,2,5,6-dibenzanthracene, and methylcholanthrene in group 4 developed atrophy of the genital organs in both sexes. These atrophic changes were most pronounced in the dibenzanthracene-fed animals. In mice receiving dibenzanthracene and injections of testosterone proprionate genital atrophy was noted in some of the animals killed early; but in the animals treated for more than 3 months, this change was apparently not so marked as in the animals getting dibenzanthracene alone. In the mice fed dibenzanthracene the genital atrophy made its appearance about the third month of treatment and was usually marked after the fifth month. Within the various groups there was some individual variation, as shown by the fact that some mice had less atrophy than others which had been ingesting emulsion for shorter periods.

In the males the prostate glands, seminal vesicles, coagulating glands, and testes were affected. The testes were a smooth, light brown and uniformly reduced in size. There appeared to be a general reduction both in the number and diameter of the tubules. Many tubules exhibited diminished spermatogenesis. There was a coincident decrease in the thickness of the wall of the tubule and increase in the diameter of the lumen. Tubules scattered sporadically through the testicle were lined by only one or two cell layers with total absence of spermatozoa (pl.1, *D*). In these tubules many lining cells were degenerated and necrotic. The number of these affected tubules varied considerably in the same and in different testes, but the process was never diffuse. The interstitial cells in certain specimens appeared to be reduced in number. Sections of the epididymis from some of the mice showed atrophy of tubules and a diminished content of spermatozoa. The seminal vesicles and coagulating glands of the affected animals were uniformly reduced in size and in some animals appeared totally atrophied so that it was difficult to find them at autopsy. The lining appeared atrophic and flattened, and the amount of secretion in the lumens of the glands was diminished. Both the ventral and posterior prostate glands became atrophic. In the ventral prostate the acini were dilated and the walls were thin, because

of decrease of the connective tissue and atrophy and flattening of the lining cells. The posterior protate showed reduction in size of the entire organ. The individual acini showed thinning and flattening of the epithelium with retraction of the papillary projections, many glands being lined by a single layer of flattened cells. In some sections the acini were contracted. One animal in group 4 which had received methylcholanthrene emulsion showed at autopsy a periurethal spindle-cell sarcoma and a second nodule of spindle-cell sarcoma embedded in the mesentery attached to the ascending colon. The relationship between these two tumors in the same mouse was not entirely clear, although the possibility was considered that the periurethal growth was primary and the mesenteric lesion a metastatic deposit.

The 10 female mice which were fed dibenzanthracene emulsion for 1 to 9 months showed atrophy of the uterus and ovaries in all cases. This atrophic process was most pronounced in the mice which ingested the emulsion for longer periods of time. There was diminution in the diameter of the uterus and disappearance of the surrounding fat. The endometrium in many cases consisted of a single layer of columnar cells with only an occasional gland in an atrophic stroma. The myometrium also appeared to be somewhat reduced in thickness (pl. 2, *A*).

### INTESTINES

Two lesions of the small intestines were observed, namely, a diffuse low-grade inflammatory lesion and adenocarcinoma.

The inflammatory lesion occurred in the experimental mice of all groups. It was distinctly less pronounced in the animals ingesting methylcholanthrene and appeared only after prolonged treatment. In the dibenzanthracene-fed mice this intestinal lesion developed within the first 2 months, and after the fifth month it was observed in pronounced form in almost all animals. The lesion was usually not found if drinking water was substituted for the dibenzanthracene emulsion a few weeks prior to autopsy. This indicated the reversibility of the lesion and its dependence upon continued ingestion of the hydrocarbons. Grossly, the lesion was characterized by a change in the appearance of the small intestine beginning abruptly in the duodenum and fading out gradually in the terminal ileum. The small intestine in the area of involvement was white, opaque, thickened, and atonic with diminution of annular contractions. In a few animals the intestinal serosa along the mesenteric attachment showed a delicate whitish network, and occasionally there were associated small petechiae. The wall of the intestine was thickened, and when opened the lumen was empty or at most contained only thin liquid. The milky white appearance was therefore due not to anything in the lumen but to material in the wall of the intestine. Two mice showed intestinal hemorrhage from an unidentified source.

Microscopically, the low-grade inflammatory lesion was characterized by marked edema, accumulation of lipoidal material, and dilatation of the lymph vessels in the stroma of the glands and in the connective tissue between the base of the glands and the muscular coat (pl. 2, *B*). There was a focal or thinly scattered diffuse infiltration of lymphocytes, plasma cells, and both mononuclear and polymorphonuclear leukocytes. In a few specimens the cell infiltration consisted chiefly of eosinophils. These cells were also occasionally present in the stroma between the glands and rarely in the musculature. The lymph follicles were frequently atrophic. In a few specimens of small intestine the base of

249431—40——3

the glands extended into the submucosa, and these glands were lined by hyperchromatic cells which were somewhat atypical in size and shape. Occasionally, small gland masses became apparently detached from the overlying mucous membrane. The Nile blue sulphate and sudan III stains were applied to frozen sections of the stomach and intestines. In the jejunum and duodenum there was a large amount of fine, stainable, lipoidal droplets in the glandular cells, in the stroma of the mucosa, and in the connective tissue in the submucosa. In the ileum the distribution of the lipoidal material was similar, except that around some of the blood vessels which traversed the musculature were fine lipoidal droplets probably in lymphatics. There was more lipoidal material in the submucosa of the ileum than in the preceding sites. Sections of the cecum, colon, and stomach contained little stainable lipoidal material.

Ten mice developed carcinoma of the small intestine. The data on these animals are given in table 3. In 7 of the animals there was a single tumor, and in each of the remaining 3 mice 2 tumors, a total of 13 in the 10 mice. Of the 13 tumors, 8 occurred at a point almost precisely 15 cm. from the pylorus, and 5 at varying distances from the pylorus as follows: 3, 6, 10, 12, and 19 cm. Since the average length of the small intestine in the strain A and strain A backcross mice is 41 cm., the majority of the intestinal tumors in this experiment occurred therefore in the jejunum. The average diameter of the tumors was 4.7 mm. They varied in size, however, from small localized plaquelike lesions 2 to 3 mm. in diameter to a more diffuse lesion 12 mm. in length. Two of the tumors were completely annular, 4 occurred on the wall of the intestine opposite the mesenteric attachment, and 6 on the side of the mesenteric attachment; in 1 this feature was not recorded. The lesions were readily observed at autopsy by external inspection of the intestine (pl. 3, *A*). They were firm, gray or whitish, elevated nodules on the serosa, encircling from one-half to the entire circumference of the intestine. Occasionally, they showed small petechiae on the external surface. The larger diffuse tumors converted the intestine in the area of involvement into a solid tube with multiple small granular excrescences on the serosal surface. In one case in which the tumor proper was of average size, the adjacent intestine was irregularly thickened for a distance of 8 mm. on either side of the lesion. In 2 cases adhesions extended from the tumor to the right testicle and to the pelvic wall. In 5 cases there were small nodules 1 mm. in diameter in the mesentery adjacent to, or leading away from the tumor. In 3 cases there were fine, gray striations extending between these nodules and the primary tumor. In 1 of these cases, a mesenteric lymph node was enlarged, firm, mottled gray and red, and contained carcinoma deposits. The segment of intestine proximal to the tumor was occasionally slightly dilated and the distal portion collapsed. In 1 case in which 2 tumors were present, the intervening intestine was dilated. Upon incising the tumor, the lumen of the intestine was frequently found constricted. This incomplete occlusion of the lumen was due partly to obstruction by the firm, thickened, elevated mucous membrane at the point of involvement, and partly to constriction by the tumor. The lumen in 1 case was represented by only a slitlike opening. Occasionally there was a small area of ulceration covered with a gray, green, or reddish exudate. In 1 case multiple small ulcers were noted at the level of the tumor and in the adjacent mucosa.

All the tumors were examined histolog-

ically. They were composed of atypical epithelial cells arranged in acinar formation, and solid masses, and nests. These atypical epithelial cells were continuous with the glandular cells of the normal mucous membrane on either side of the tumor. They infiltrated the entire wall of the intestine and in all cases formed an elevated nodule on the serosal surface (pl. 3, *B* and *C*). In a number of cases the serosal nodule was embedded in the mesentery. The growth caused pronounced thickening of the intestinal wall in the area of involvement. In a few cases, the mucous membrane and underlying musculature were thickened in their entire circumference owing to permeation by atypical epithelial cells. In those cases in which only a segment of the intestines was involved by tumor, the remaining mucous membrane except at the line of demarcation appeared fairly normal. In five cases ulceration was shown microscopically. The base of the ulcer was composed of tumor and granulation tissues. In the case with multiple ulcers in the adjoining mucosa the base was formed by inflammatory cells. In several cases there were small abscesslike inflammatory lesions in the wall of the intestine and in the mesentery. In all cases the entire musculature and other tissues were destroyed in the area infiltrated by the tumor. In the adjoining surviving musculature the fibers were separated by infiltrating tumor tissue which also extended somewhat laterally in the submucosa. The mesentery was frequently infiltrated by tumor tissue for an appreciable distance beyond its attachment to the intestine (pl. 4).

The glands forming the tumor resembled, in general, the elongated glands of the intestine. In all specimens variation in the glands was noted. Some were round, irregularly shaped, small, cystic, or imperfectly formed with papillary formation. In all the tumors some of the atypical cells grew in the forms of nests. The lumens of the glands frequently contained necrotic material and masses of minute acidophilic refractive granules. Similar granules were also present in secretion vacuoles in some tumor cells and also in some of the epithelial cells of the unchanged mucous membrane.

The tumor cells lining the glands and occurring singly in the nests and in groups resembled the normal glandular epithelium of the intestinal mucous membrane. They were hyperchromatic columnar cells without much variation in size and shape; in the acini they were frequently stratified. Some departure from this general appearance was noted in all the tumors, however, so that some cells were cuboidal, and others flattened and irregular. Mitotic figures averaged 1 to 4 per high-power field in all but two of the tumors in which they were less frequent. In one case there was marked individual variation in the tumor cells, the majority of them occurring in nests and small groups embedded in connective tissue. In several cases small droplets of mucin occurred in the tumor cells and distended the lumens of some of the glands. One tumor was essentially a mucinous carcinoma, composed of a moderate number of mucin-filled acini, the bulk of the tumor consisting of masses and nests of round cells surrounded by strands of mucin embedded in connective tissue. Most of these cells contained vacuoles filled with mucin which so distended the cell as to displace the nucleus to one side.

The line of demarcation between the normal mucous membrane and the tumor was usually abrupt (pl. 5, *A*). The first change noted on approaching the tumor from the sound mucous membrane was the presence of irregular glands and masses of atypical cells scattered about in

the mucous membrane near its junction with the tumor, which caused thickening and elevation. In the ulcerated lesions the atypical cells forming the base of the ulcers were in continuity with similar cells in the adjoining mucosa. The deep portions of the glands in the more normal mucous membrane showed hyperchromatic cells and a limited tendency to infiltrate the immediately adjacent submucosa. In one case the entire circumference of the mucous membrane was composed of atypical glands. The amount of stroma varied in the different tumors. In half of them it was relatively scanty, whereas it was abundant in the remainder; three tumors were of the scirrhous type. The stroma was composed of adult connective tissue frequently showing inflammatory cell infiltration. In most cases the blood vessels were not numerous, and all were well formed. The tumors were well preserved except for a few areas of necrosis and hemorrhage.

Microscopic examination of the mesenteric lymph nodes from the mice with intestinal carcinoma revealed the presence of inflammatory hyperplasia in several. One of these specimens also showed metastatic deposits from the primary tumor in the intestine (pl. 5, *B*). The lymph node from this case showed congestion, hemorrhage, dilatation of lymph sinuses, and extensive replacement of the pulp and peripheral sinuses by tumor tissue. The metastatic deposits consisted of columnar, cuboidal, and flattened atypical cells. Some of these were arranged in acini, and others in solid masses and nests. The appearance of the metastatic lesion closely resembled that of the primary tumor in cell type and arrangement. A large amount of stroma and numerous areas of hemorrhage and necrosis were present.

Three of the intestinal tumors were transplanted. In obtaining the transplant small fragments of the primary tumor were excised and immediately transferred by means of trocar and cannula to the subcutaneous tissue of other mice of the same strain as the animal in which the tumor arose. Three recipient mice were used in each case. The transplant from only one case was successful and is now in the third generation. The failure of the other transplants to grow was considered due in part to the fact that the transplanted material was necessarily infected and that the transplants consisted of exceedingly small fragments of tissue. Microscopic examination of the successful transplants showed a cell type and arrangement similar to those of the primary tumor from which the transplants were obtained.

### MISCELLANEOUS FINDINGS

The pancreas in a few strain A mice ingesting dibenzanthracene showed diffuse infiltration of inflammatory cells in the connective tissue stroma of the gland. One strain A female mouse fed dibenzanthracene developed a transitional cell carcinoma in the region of the salivary glands. Since tumors of this type and location are occasionally observed in strain A mice not subjected to any treatment, the tumor in this instance could not be considered related to the experimental procedure. A strain A backcross mouse of group 5 developed a spindle-cell sarcoma of the chest wall. No explanation for this is at hand. No particular change was noted in the pituitary, thyroid, or the adrenal glands of the experimental or control mice with the staining techniques employed. The adrenal glands were occasionally smaller than usual but showed no specific cellular change. In the mice receiving injections of testosterone proprionate and sesame oil collections of this oily material were frequently found in the subcutaneous tissue

and in other sites into which the injections had been made. These encysted oil areas were sometimes associated with local infection.

## DISCUSSION

The results of these experiments indicate that mice can ingest large amounts of dibenzanthracene and methylcholanthrene, considerably in excess of the amounts necessary for tumor induction by skin painting or subcutaneous injection. The maximum amount of dibenzanthracene that the mice were capable of ingesting and tolerating was of the order of magnitude of 160 to 190 mg., although emulsions of different hydrocarbon content were used and the daily average intake varied considerably in the different experiments. Methylcholanthrene on the other hand was better tolerated, as shown by the fact that the maximum amount ingested by a single mouse was 275 mg. The data obtained by absorption spectrum analysis of the tissues, body fluids, and excreta of mice ingesting dibenzanthracene revealed that the hydrocarbon was present in unchanged form in the gastrointestinal tract down to the level of the ileocecal junction. In the lung, liver, kidney, blood, subcutaneous fat, and ascitic fluid no hydrocarbon was found within the limit of sensitivity of the spectrographic method, except in the case of two pairs of lungs. The presence of dibenzanthracene in these lungs is believed to represent a contamination. No spectrographic data are available as yet for the organs and tissues of the mice ingesting methylcholanthrene.

The fact that the experimental animals regularly developed multiple, primary pulmonary tumors after a few months of oral administration of dibenzanthracene and methylcholanthrene leads to the assumption that the hydrocarbon gained access to the lung tissue by absorption from the gastrointestinal tract. These tumors corresponded in number, distribution, and structure to the pulmonary tumors induced by the subcutaneous, or intravenous injection of the hydrocarbon (Grady and Stewart (*8*)). Magnus (*6*) also reported the production of multiple pulmonary tumors in mice receiving dibenzanthracene in olive oil by stomach tube. He considered it highly probable that his findings resulted from aspiration of minute quantities of the carcinogenic agent. Since in the experiment described here, aspiration of minute quantities of the emulsion might conceivably have occurred during drinking, a group of seven mice were given a charcoal suspension in drinking water containing 1 mg. of charcoal per cubic centimeter for 4 months. Numerous histologic sections of the lungs of each of these animals failed to disclose the presence of any charcoal particles. Therefore aspiration of the emulsions during the natural act of drinking appears improbable and not responsible for the production of lung tumors in the experimental animals. To rule out the possibility that these lung tumors were induced by inhalation of spilled emulsions, five untreated control mice were kept from 8 to 12 months on sawdust mixed weekly with 20 cc. of aqueous olive-oil emulsion containing 8 mg. of dibenzanthracene. No lung tumors were induced by this method. Likewise, the animals given aqueous olive-oil emulsion showed no induced pulmonary tumors.

This evidence favors the assumption that the hydrocarbon reached the lungs of the experimental mice by absorption from the gastrointestinal tract, and implies that it was also present in the blood. As the gastrointestinal tract is relatively resistant to tumor induction, the method of oral administration can be employed to keep the hydrocarbon in the system of

experimental animals over long periods of time, thus permitting study of the progressive effects of hydrocarbons on the organs and tissues. The advantages of this method over intravenous or parenteral injection of, or skin painting with the hydrocarbon are apparent. Under the latter conditions either the experiment is terminated by tumor production at the site of application; or in the case of intravenous injection, the hydrocarbon is rapidly eliminated from the system.[7]

The general systemic effects aside from from the production of tumors have been described in the section on pathological observations. In brief, these consisted of emaciation, premature aging, anasarca, and atrophic changes in certain organs and tissues. It follows from the data in table 1 that for equal amounts administered and for mice of the same genetic constitution, dibenzanthracene is more pronounced in its apparent toxic effects on the organs and tissues than the carcinogenically more potent methylcholanthrene. From curve 1 it seems to follow that the feeding of dibenzanthracene retards the normal gain in weight. This is in line with the findings of Haddow (9) and Lee (10). However, this retardation in weight gain might be due not entirely to the direct effect of the hydrocarbon on the organism, but also among other things to the effects of fluid deprivation, since these mice drank only 2.5 cc. per day of the dibenzanthracene emulsion compared with 4.5 cc. of plain emulsion and 5 to 6 cc. of water, ingested by the controls. In an attempt to counteract the effects of atrophy on the male genital organs in the mice ingesting dibenzanthracene, the mice of group 5 were given simultaneously testosterone proprionate in sesame oil by subcutaneous injection. It was hoped to prolong the experimental period by this treatment. Although an influence of the hormone on the genital atrophy could apparently be demonstrated, the dose given proved insufficient, and besides, infection of the tissue about the oil pockets at the sites of injection proved to be an unfavorable factor.

The most important result in these experiments was the induction of intestinal carcinoma in the mice of groups 3 and 4. Spontaneous carcinoma of the small intestine of mice is exceedingly rare. Murray (*11*) described an adenocarcinoma of the small intestine with characteristic infiltration of the gut wall and mentioned a similar case reported by Twort. A lesion of the ileum, reported by Heidenhain (*12*), showed atypical glandlike structures in the submucosa but no infiltration of the musculature. Löwenthal (*13*) speaks of a chronic duodenal ulcer or duodenal carcinoma in one of a series of mice injected with embryo or placental extracts but gives no more adequate description. Wells, Slye, and Holmes (*14*) found no case of carcinoma of the small intestine in 142,000 autopsies on mice. Likewise in many thousand autopsies performed on mice in this laboratory, no case of a spontaneous tumor of the small intestine has been observed. Also the controls to the experimental mice in this study showed no intestinal carcinoma.

Since all the mice in this series had multiple tumors of the lung, the question arose whether the intestinal neoplasms represented metastatic tumors from the primary growths in the lung. It would be extremely unusual for metastasis from multiple pulmonary tumors to be restricted to one or two localized areas of the small intestines in these experimental animals and not involve other sites. Pulmonary tumors which yielded intestinal metastasis have not been observed in this laboratory.

[7] *Unpublished data obtained by absorption spectrum analysis.*

Furthermore, the microscopic appearance of the intestinal tumors was suggestive of primary intestinal neoplasms in cell type and cell arrangement; several of the neoplasms contained mucin; and many of the cells were filled with highly refractive acidophilic granules resembling those normally found in the cells lining the crypts of Lieberkühn in the intestinal mucous membrane. These features are not characteristic of primary pulmonary tumors of the mouse.

The intestinal carcinomas in the present study were observed in the animals of experimental groups 3 and 4. In group 3 strain A mice were given the dibenzanthracene emulsion, while in group 4 strain A backcross mice were given the methylcholanthrene emulsion. The animals of the other experimental groups were strain A backcross mice receiving the dibenzanthracene emulsion. No intestinal tumors were observed in these latter groups although the maximum amount of dibenzanthracene ingested by many of the strain A backcross mice was at least of the same order of magnitude as that ingested by the strain A mice which did develop intestinal tumors. This failure to obtain intestinal tumors may be explained on the basis that the strain A backcross mice were apparently more resistant to the induction of intestinal tumors by dibenzanthracene than strain A mice but did develop this lesion when the more potent methylcholanthrene was given.

All the 13 intestinal carcinomas occurred in the upper small intestine. No tumors developed in any other part of the gastrointestinal tract, although the hydrocarbon was present down to the level of the large intestine. It is difficult to explain why tumor induction in the alimentary canal was restricted to the upper small intestines. Several factors may have favored tumor induction in this location. The exposure of the upper gastrointestinal tract (mouth and esophagus) to the hydrocarbon was intermittent. Besides, there was probably little contact of the carcinogen with the tissue, as the carcinogenic component of the emulsion was contained in the oil droplets which were surrounded by a soap film. In the stomach the emulsion was mixed with food and probably partly broken by the acidity of that viscus. It can be assumed that digestion of the olive oil began in the stomach and was completed in the small intestine. Following digestion of the olive oil, the hydrocarbon was probably liberated in the form of a dispersion which could then act directly upon the cells of the intestinal mucous membrane. In the large intestines no dibenzanthracene was present as such, but a new compound apparently related to it was found.

Besides the intestinal carcinomas, a low-grade inflammatory lesion was observed in the wall of the small intestine, which was associated with edema and accumulation of large amounts of lipoidal material. This lesion was most pronounced in the mice ingesting the dibenzanthracene in which it was observed within the first 2 months and in pronounced form after the fifth month. With this treatment the lesion was equally pronounced in the strain A backcross and strain A mice although only the strain A mice developed intestinal carcinoma. In the mice ingesting methylcholanthrene this lesion was distinctly less pronounced and appeared only after prolonged treatment. The lesion was dependent upon the ingestion of the hydrocarbons, as control mice receiving plain olive-oil emulsion or drinking water did not develop it. Moreover, it disappeared in the experimental mice which were deprived of the hydrocarbon and given drinking water a few weeks prior to autopsy. The pathogenesis

of this process is obscure, as is also its relationship to the development of intestinal carcinoma.

Some relationship apparently existed between this low-grade inflammatory lesion of the intestine and the development of ascites. The mice ingesting dibenzanthracene developed both lesions earlier in the experiment and more frequently and more severely than the mice receiving methylcholanthrene. The ascites also disappeared when drinking water was substituted for the emulsion containing hydrocarbons. If the animals with ascites were allowed unrestricted access to drinking water, there was often a rapid accumulation of peritoneal fluid causing death. This phenomenon also occurred in some experimental mice in which the ascites was minimal or undetectable clinically, when they were allowed unrestricted access to water. However, if after the deprivation of the hydrocarbon the amount of drinking water allowed the animals was restricted, the ascites usually disappeared and the mice could then again be given the hydrocarbon emulsions. Although the ascitic fluid contained either microscopic droplets of oil or was frankly chylous, repeated absorption spectrum analysis failed to disclose the presence of the hydrocarbon (dibenzanthracene). No studies were undertaken to determine the mechanism of the development of this ascites.

Likewise, no explanation is at hand for the selective effect of the hydrocarbons on certain tissues and organs of the body. As a result of injections of carcinogenic hydrocarbons into rodents, changes in certain organs and tissues distant from the site of injection have been reported (Picard (*15*) as reported by Cook and Kennaway (*16*), Nakahara and Fujiwara (*17*, *18*), Parsons (*19*), Csatro, Wetzler-Ligeti and Wiesner (*20*)). These changes consisted of degeneration of thyroid and ovaries, ascites and atrophy of spleen, lymph nodes, thymus, and bone marrow. While in our experiments the hematopoietic tissues and the genital organs were severely affected, other organs showed little or no change aside from those associated with emaciation. Again the effects produced by ingestion of dibenzanthracene were more pronounced in this respect than those produced by methylcholanthrene although the ultimate pathological changes were of a parallel nature. A point of interest is the absence of any pronounced change of the kidneys in the experimental animals of all groups although according to Dobriner, Rhoads, and Lavin (*21*) a conversion product of dibenzanthracene is excreted in the urine of animals treated with the hydrocarbons. The livers, also, showed relatively little change except for a few cases of focal bile-duct proliferation or focal necrosis and one case of minimal cirrhosis. This is in contrast to findings in rabbits (Claude (*22*), Polson (*23*)), guinea pigs (Shabad (*24*)), and rats (Blumberg and Grady,[8] Goerner and Goerner (*25*)) in which pronounced liver damage may result from treatment with dibenzanthracene or methylcholanthrene. This suggests a species difference in the manner in which the liver tissue reacts to these compounds.

In an attempt to explain the atrophic changes in the genital organs, the pituitary glands of most of the mice of groups 3, 4, 5, and 6 were studied. They were removed at autopsy under a dissecting microscope, fixed in Zenker's fluid, blocked in paraffin, cut in complete serial sections, and stained with Mallory's anilin-blue stain. In no case was there an adenoma or any other lesion of the pituitary observed. However, this technique was

[8] *Blumberg, H., and Grady, H. G.: Personal communication.*

wholly inadequate for differential cell staining of the pituitary cells of the mouse. This feature awaits further studies.

## SUMMARY AND CONCLUSIONS

1. Strain A and strain A backcross mice were given orally aqueous olive-oil emulsions containing either 1,2,5,6-dibenzanthracene or 20-methylcholanthrene instead of drinking water, for various periods of time up to 13 months.

2. The method for preparation and administration of the emulsions is described.

3. The fate of the dibenzanthracene in the body tissue, the body fluids, and excreta was traced by absorption spectrum analysis. It was found in unchanged form in the gastrointestinal tract to the level of the ileocecal junction. No dibenzanthracene was found in the large intestine and the feces, and none was detected in other body tissues and fluids within the limit of sensitivity of the spectrographic method. There is presumptive evidence, however, that absorption of the hydrocarbon from the gastrointestinal tract occurs, which explains tumor induction in the lung.

4. The principal lesions observed were adenocarcinoma of the small intestine, multiple primary tumors of the lung, atrophy of the hematopoietic and genital tissues, and anasarca.

## REFERENCES

(*1*) Reinhard, M. C., and Candee, C. F.: Development of multiple tumors in mice: Part III. Results of ingestion of carcinogenic agents: Preliminary report. Am. J. Cancer, **26:** 552–553 (1936).

(*2*) Oberling, C., Sannié, C., and Guerin, M. and P.: Recherches sur l'action cancérigène du 1, 2-benzopyrène. Bull. Assoc. franc. p. l'étude du cancer, **25:** 156–180 (1936).

(*3*) Cook, J. W., Haslewood, G. A. D., Hewett, C. L., Hieger, I., Kennaway, E. L., and Mayneord, W. V.: Chemical compounds as carcinogenic agents. Am. J. Cancer, **29:** 219–259 (1937).

(*4*) Waterman, N.: Experimental production of carcinoma in the stomach of mice. Acta Cancrol., **2:** 375–388 (1936).

(*5*) Van Prohaska, J., Brunschwig, A., and Wilson, H.: Oral administration of methylcholanthrene to mice. Arch. Surg., **38:** 328–333 (1939).

(*6*) Magnus, H. A.: The experimental production of malignant papillomata of the lung in mice with 1:2:5:6-dibenzanthracene. J. Path. & Bact., **49:** 21–31 (1939).

(*7*) Lorenz, E., and Shear, M. J.: Studies in carcinogenesis. II. The detection of dibenzanthracene in mouse tumors induced by this hydrocarbon. Am. J. Cancer, **26:** 333–343 (1936).

(*8*) Grady, H. G., and Stewart, H. L.: Histogenesis of induced pulmonary tumors in strain A mice. Am. J. Path., **16:** 417–432 (1940).

(*9*) Haddow, A., Scott, C. M., and Scott, I. D.: The influence of certain carcinogenic and other hydrocarbons on body growth in the rat. Proc. Roy. Soc. London, s. B, **122:** 477–507 (1937).

(*10*) Lee, J. C.: The inhibition of growth by 1, 2, 5, 6-dibenzanthracene and other agents. Quart. J. Exper. Physiol., **27:** 162–170 (1937).

(*11*) Murray, J. A.: Spontaneous tumor in the mouse; histology, metastasis, transplantability and the relations of malignant new growths to spontaneously affected animals. [Great Brit.] Imp. Cancer Research Fund, 3d Scient. Rep., pp. 69–114 (1908).

(*12*) Heidenhain, K.: Eine seltene Geschwulst des Blinddarms bei einer Impfmaus. Ein weitere Beitrag zur Morphogenese. Virchows Arch. f. path. Anat., **273:** 541–552 (1929).

(*13*) Löwenthal, K.: Einige Grundfragen der experimentellen Geschwulstforschung. Med. Klin., **24:** 1263–1268 (1928).

(*14*) Welles, H. G., Slye, M., and Holmes, H. F.: Comparative pathology of cancer of the alimentary canal, with report of cases in mice; studies in incidence and in-

249431—40——4

heritability of spontaneous tumors in mice. Am. J. Cancer, **33:** 223–238 (1938).

(*15*) PICARD, E.: 2d Internat. Cancer Cong., Brussels, vol. 2, p. 120 (1936). Cited by Cook, J. W., and Kennaway, E. L.

(*16*) COOK, J. W., and KENNAWAY, E. L.: Chemical compounds as carcinogenic agents. First supplementary report: Literature of 1937. Am. J. Cancer, **33:** 50–97 (1937). See p. 74.

(*17*) NAKAHARA, W., and FUJIWARA, T.: Carcinogenesis experiments based on intraperitoneal injections; 3,4-benzpyrene. Gann, **31:** 568–574 (1937).

(*18*) NAKAHARA, W., and FUJIWARA, T.: Carcinogenesis experiments based on intraperitoneal injections; methylcholanthrene. Gann, **31:** 660–663 (1937).

(*19*) PARSONS, L. D.: Changes in the lymph glands of tumour-bearing mice. J. Path. & Bact., **47:** 501–523 (1938).

(*20*) CSATO, T., WETZLER-LIGETI, C., and WIESNER, B. P.: Effect of the prolonged administration of 1 : 2 : 5 : 6-dibenzanthracene on the reticulo-endothelial system of rabbits. Am. J. Cancer, **36:** 257–261 (1939).

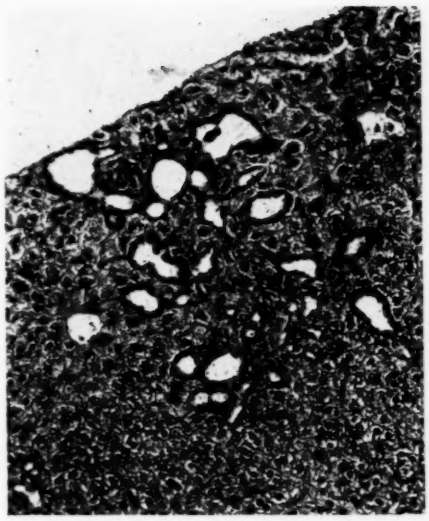
(*21*) DOBRINER, K., RHOADS, C. P., and LAVIN, G. I.: Conversion of 1, 2, 5, 6-dibenzanthracene by rabbits, rats, and mice. Significance in carcinogenesis of this conversion. Proc. Soc. Exper. Biol. & Med., **41:** 67–69 (1939).

(*22*) CLAUDE, A.: Liver degeneration and cirrhosis produced by 1 : 2 : 5 : 6-dibenzanthracene. Am. J. Cancer, **31:** 100–103 (1937).

(*23*) POLSON, C.: Experimental liver necrosis produced by 1 : 2 : 5 : 6-dibenzanthracene. J. Path. & Bact., **42:** 537–538 (1936).

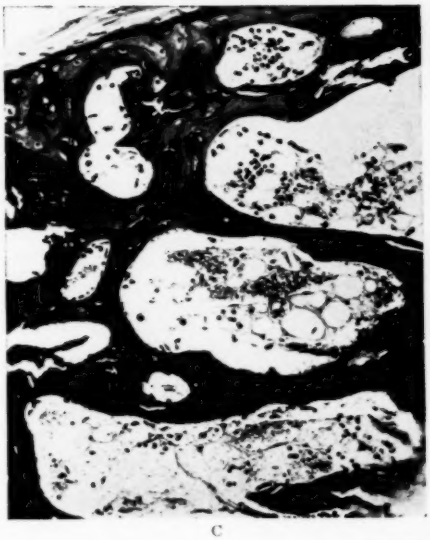
(*24*) SHABAD, L. M., and URINSON, J. P.: Alterations in the liver of guinea-pigs following administration of a chemically pure carcinogenic compound (1 : 2 : 5 : 6-dibenzanthracene.) Arch. des Sci. biol. (U. S. S. R.), **51:** 105–111 (1938). [In Russian. English summary, p. 111.]

(*25*) GOERNER, A., and GOERNER, M. M.: The effect of dibenzanthracene on the liver. Am. J. Cancer, **37:** 518–520 (1939).



A

B



C

D

LEGEND FOR PLATE 1

*A*, Focal area of bile duct proliferation and fibrosis near the surface of the liver. × 170. *B*, Portion of the spleen showing atrophy of the follicles and pulp cells causing shrinking of the organ, thereby allowing the trabeculae to come into close apposition to each other. There is a pronounced accumulation of pigment. × 167. *C*, Portion of the scapula, showing marked depletion of bone marrow cells and replacement of the marrow cavities by fat and congested blood vessels. × 235. *D*, Portion of the testicle. The tubule in the center has a lining only one or two cells thick, and there is an almost complete absence of spermatogenesis. The lumen of the tubule contains degenerated and necrotic cells. × 495.

LEGEND FOR PLATE 2

*A*, Atrophy of the uterine glands and the endometrium. The lining of the uterine cavity consists essentially of a single layer of columnar cells. × 112. *B*, Submucosa of the small intestine is wider than normal, and pale owing to the presence of fat, edema, and dilated lymphatic vessels. The deeper portions of the glands are lined by hyperchromatic cells. × 30.

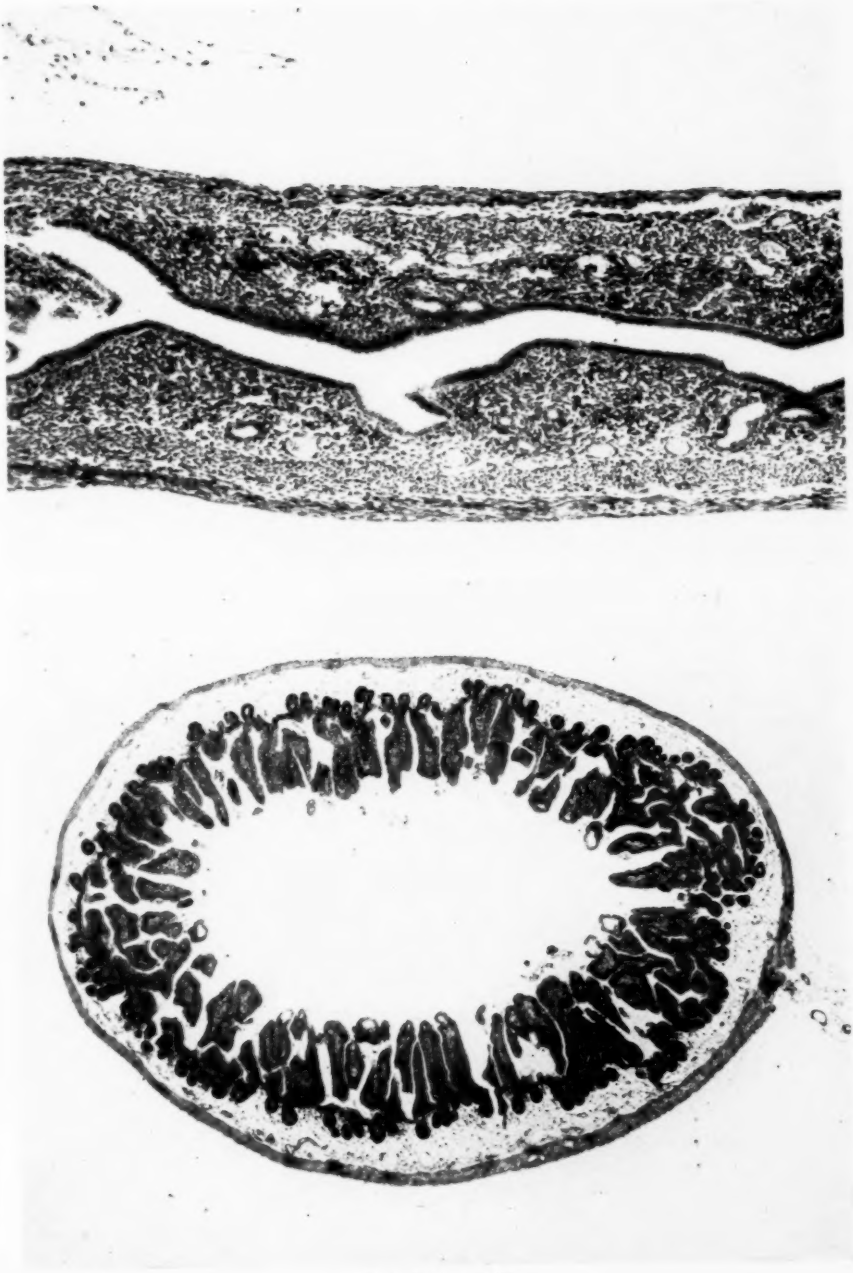
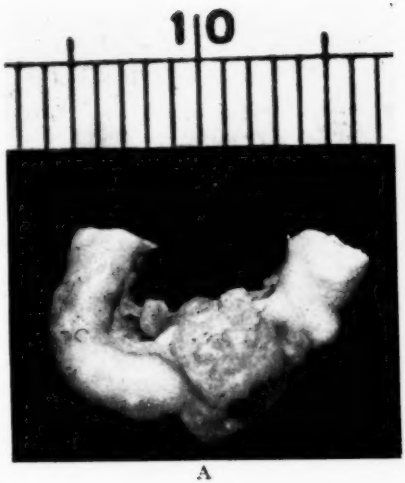


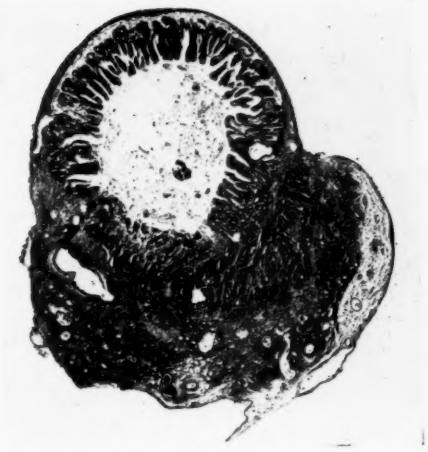
Plate 2 (A and B)

LEGEND FOR PLATE 3

*A*, Portion of the small intestine, showing firm annular carcinoma which forms an elevated nodule on the serosal surface. Photograph of gross specimen enlarged about 3.8 times. *B*, Mucous membrane lining half of the circumference of the small intestine appears normal. In lower half the glands of the mucous membrane are interspersed with atypical gland structures that permeate the underlying submucosa and musculature and form an elevated mass on the serosa. At the extreme lower margin can be seen a small tab of mesentery. This illustrates the localized type of tumor. × 13. *C*, Lining of the mucous surface of the small intestine is replaced in all but one small area at the lower margin by atypical cells which infiltrate through all coats of the intestine. This illustrates the annular tumor. × 13.




A



B

C

LEGEND FOR PLATE 4

Small intestine and mesenteric attachment showing a nodular mass on the serosal surface, composed of atypical glands which infiltrate the mesentery. × 87.

PLATE 4.

LEGEND FOR PLATE 5

*A*, Small intestine showing junction between normal mucosa and a nodule of adenocarcinoma. The carcinoma is composed of atypical glands, some markedly dilated, which permeate all coats of the intestinal wall. × 39. *B*, Mesenteric lymph node containing metastatic deposits of an adenocarcinoma of small intestine. × 143.

PLATE 5.

# RELATIVE IMPORTANCE OF LOCAL AND CONSTITUTIONAL EFFECTS OF METHYLCHOLANTHRENE IN PRODUCTION OF SKIN TUMORS IN THE MOUSE [1]

By G. BURROUGHS MIDER, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service,* and JOHN J. MORTON, *professor of surgery, Department of Surgery, University of Rochester School of Medicine and Dentistry*

Spontaneous epithelial skin tumors are almost unknown in mice less than 1 year old. Multiple paintings with a benzene extract of certain coal tars on the same area of skin will produce papillomata at the site of application. Some of these eventually become squamous-cell carcinomata. The extensive literature on tar cancer in mice has been ably summarized by Woglom (*1*) and by Seelig and Cooper (*2*).

There are almost as many explanations of the way in which tar produces a malignant growth as there are investigators. The principal argument has centered, however, about the question whether it brings about a constitutional change of which cancer is but the local manifestation (*1*). The solution of this problem remains an important objective of cancer research.

Earlier investigations were carried out necessarily with market mice. They represented a heterogeneous population differing widely in their reactions to a given stimulus. Tars and tar extracts varied in their capacity for producing tumors. The development of inbred strains of mice and polycyclic carcinogenic hydrocarbons has provided two new tools for cancer in-investigation which should reduce experimental variation. The investigation reported herein is an attempt to determine whether the local or constitutional action of a carcinogenic agent is more important in the production of mouse skin tumors.

Both C57 black and C57 brown mice were isolated from a single stock about 1925.[2] [3] They have been maintained by brother-sister mating ever since and retain their distinguishing characteristics. They should be regarded as two distinct strains despite the similarity in name.

C57 black mice (*3*) are intense black and nonagouti. They have less than 1 percent incidence of spontaneous epithelial tumors. Nonepithelial neoplasms, chiefly "lymphoblastomata," occur in 19.5 percent of the virgin females and in 17.8 percent of the males. No tumors appear in appreciable number during the first year of life. The strain is an excellent test object for the study of skin- and subcutaneous-tumor induction by polycyclic hydrocarbons.

C57 brown mice [4] are chocolate brown, nonagouti, and frequently have a ventral white spot. Mammary tumors occur in about 5 percent of the breeding females. The incidence of nonepithelial neoplasms approximates that of the C57 black strain.

A 0.5-percent solution of methylcholanthrene [5] in benzene was used as the carcinogenic agent. It was applied to the unepilated skin with a No. 6 camel's-hair brush.

[1] *This work was done at the Department of Surgery, University of Rochester School of Medicine and Dentistry, and was aided in part by a grant from International Cancer Research Foundation.*

[2] *All mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.*

[3] *Bittner, J. J.: Personal communication.*

[4] *See footnote 3.*

[5] *Methylcholanthrene was synthesized by Dr. W. E. Bruce, Cornell University, Ithaca, N. Y., to whom we express our appreciation.*

The mice were kept in glass cages. They were 6 weeks old when first painted. Sex distribution was approximately equal, but breeding was prevented. The diet consisted of Purina dog chow, water being available at all times.

Each tumor that arose was individually identified and its life history recorded. Malignant change in a papilloma was indicated by alteration in the growth rate of the tumor, by ulceration, and, most important, by induration at the base. All diagnoses of carcinoma were confirmed histologically, invasion of muscle being adopted as the criterion of malignancy.

The occurrence of epithelial skin tumors in C57 brown mice after a single application of methylcholanthrene was reported previously (*4*). These papillomata are morphologically identical with those arising at the site of multiple paintings. They differ biologically. Few papillomata regress when painting is continued twice weekly, and malignancy commonly supervenes. Spontaneous regression is the rule and malignant transformation the exception, among papillomata that arise after a single application of methylcholanthrene.

Fifty C57 black mice were painted in the interscapular region twice weekly for 12 weeks with a 0.5-percent solution of methylcholanthrene in benzene. The mice were examined twice a week until the one hundred and sixty-fifth day after the initial painting when all survivors were killed. Thirty C57 black mice were painted only once from sacrum to occiput with the same solution. They were then observed until natural death. Skin-tumor production in the two groups is summarized in table 1.

The incidence of mice with papillomata and the total number of papillomata were much less in the group that received a single painting. Of the seven papillomata that arose, three disappeared within 21 days; the other four persisted for 150 to 250 days, gradually shrivelled, and dropped off. No malignancy resulted.

TABLE 1.—*Skin tumor production in C57 black mice following single or multiple paintings with a 0.5-percent solution of methylcholanthrene in benzene*

| Factor | Single painting | Multiple paintings |
|---|---|---|
| Papillomata: | | |
| Effective total | 28 | 48 |
| Mice with papilloma....number | 7 | 38 |
| Do.....percent | 25 | 79 |
| Total papillomata.....number | 7 | 112 |
| Papillomata per mouse mean number | 0.25 | 2.33 |
| Carcinomata: | | |
| Effective total | 28 | 43 |
| Mice with carcinoma....number | 0 | 15 |
| Do.....percent | 0 | 35 |
| Total carcinomata.....number | 0 | 18 |

Most of the mice that had multiple paintings developed papillomata. Only 5 papillomata disappeared during the first 120 days of the experiment. Mortality and carcinoma incidence increased markedly at the end of this time. Only 19 mice in the group survived the entire experiment. Most of those that died had carcinomata. The ultimate fate of the papillomata that did not become malignant could not be determined. Methylcholanthrene in benzene is quite toxic. The mice that survived the experimental period looked ill. The spleens and livers were enlarged at necropsy. The histologic picture resembled nonmalignant extramedullary myelopoiesis as described by Barnes and Sisman (*5*). Myeloid infiltrations, in which mature forms predominated, were pronounced in the liver, spleen, and lymph nodes. Erythrogenic foci and megakaryocytes intermingled with myeloid elements. The condition was not transplantable to other mice of the same strain. C57 black mice that received a single painting of methylcholanthrene retained their healthy appearance. Myeloid infiltrations were not conspicuous in histologic sections of their organs.

TABLE 2.—*The effect of additional methylcholanthrene on C57 brown mice bearing skin papillomata produced by a single painting of methylcholanthrene solution*

| Factor | | Single painting+multiple paintings | | | Single painting+intraperitoneal injection | | | Single painting only | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Male | Female | Total | Male | Female | Total | Male | Female | Total |
| Mice | number | 54 | 34 | 88 | 24 | 24 | 48 | 73 | 83 | 156 |
| Mice with papilloma | number | 20 | 14 | 34 | 11 | 10 | 21 | 31 | 31 | 62 |
| | percent | 37.0 | 41.2 | 38.6 | 45.8 | 41.7 | 43.7 | 42.5 | 37.3 | 39.7 |
| Total papillomata | number | 29 | 18 | 47 | 12 | 11 | 23 | 45 | 56 | 101 |
| Papillomata disappearing spontaneously | number | 20 | 11 | 31 | 8 | 8 | 16 | 32 | 37 | 69 |
| Regression | percent | 70.0 | 61.1 | 66.0 | 66.7 | 72.7 | 69.6 | 71.1 | 66.1 | 68.3 |
| Total carcinomata | number | 1 | 3 | 4 | 1 | 1 | 2 | 3 | 4 | 7 |
| All papillomata that became carcinomata | percent | 3.4 | 16.7 | 8.5 | 8.3 | 9.1 | 8.7 | 6.7 | 7.1 | 6.9 |
| Persisting papillomata that became carcinomata | percent | 11.1 | 42.9 | 25.0 | 25.0 | 33.3 | 28.6 | 23.1 | 21.0 | 21.9 |

The difference in biologic behavior of the papillomata that followed a single application of methylcholanthrene to the skin of C57 black mice is essentially the same as that found in the C57 brown strain. The additional paintings that the other group of C57 black mice received are probably responsible for the increase in production of benign and malignant skin tumors. Is the action of the additional paintings directed at the site of painting? Does it alter the physiology of the whole organism in such a manner as to predispose the benign tumor to malignant transformation?

C57 brown mice were painted once from sacrum to occiput with a 0.5-percent solution of methylcholanthrene in benzene. Thereafter they were painted twice weekly at four successive sites about the hind quarters with the same solution. The order of painting was: Left hind leg, right hind leg, base of tail, suprapubic region. Painting was continued until death. Another group of C57 brown mice was painted once from sacrum to occiput. Four weeks later, when the first papillomata appeared, they were given one injection of 0.5 cc. of sesame oil containing 2.0 mg. of methlycholanthrene. Other mice of the same strain received only the single painting with methylcholanthrene. C57 brown mice were used because they developed more skin tumors after a single painting with methylcholanthrene than did the C57 black strain. The results of this experiment appear in table 2.

No significant difference in the incidence of benign or malignant skin tumors was found among the three groups. The additional paintings with methylcholanthrene at a distance from the tumor site did not alter the biologic behavior of the papillomata. The mice that received additional paintings of the carcinogenic solution lived for 180–250 days. Carcinomata occurred between the one hundred and fourth and the two hundred and twenty-eighth day (average, 139.7 days) after painting in those mice that were painted once. The mice that were injected intraperitoneally lived at least 180 days. No intraperitoneal tumors were obtained. Two mice, however, developed a leukosis to which the strain is spontaneously susceptible.

Extramedullary myelopoiesis was found in most of the mice that received additional paintings with methylcholanthrene. It was not present in the other groups. Hence, it may not be considered of significance in the malignant transformation of papillomata in mice.

The results of these experiments suggest

that multiple paintings of this carcinogenic agent at the same site increase the incidence of benign and malignant skin tumors through local action at the site of application.

## SUMMARY

A single application of a 0.5-percent solution of methylcholanthrene in benzene to the backs of C57 black mice produced papillomata which disappeared spontaneously. Multiple paintings with the same solution produced papillomata that persisted. Some of these became malignant.

A few carcinomata occurred among the papillomata that followed a single application of a 0.5-percent solution of methylcholanthrene in benzene to the backs of C57 brown mice. Additional paintings with the same solution about the hind quarters did not affect the incidence or behavior of skin tumors arising on the back.

## REFERENCES

(*1*) Woglom, W. H.: Experimental tar cancer. Arch. Path., **2:** 533–576, 709–752 (1926).

(*2*) Seelig, M. G., and Cooper, Z. D.: A review of the recent literature of tar cancer (1927–1931, inclusive). Am. J. Cancer, **17:** 589–667 (1933).

(*3*) Little, C. C., Murray, W. S., and Cloudman, A. M.: The genetics of non-epithelial tumor formation in mice. Am. J. Cancer, **36:** 431–450 (1939).

(*4*) Mider, G. B., and Morton, J. J.: Skin tumors following a single application of methylcholanthrene in C57 brown mice. Am. J. Path., **15:** 299–302 (1939).

(*5*) Barnes, W. A., and Sisman, I. E.: Myeloid leukemia and non-malignant extramedullary myelopoiesis in mice. Am. J. Cancer, **37:** 1–35 (1939).

# STUDIES IN CARCINOGENESIS. XII. EFFECT OF THE BASIC FRACTION OF CREOSOTE OIL ON THE PRODUCTION OF TUMORS IN MICE BY CHEMICAL CARCINOGENS [1]

By ROBERT D. SALL, *student, Harvard Medical School,* and M. J. SHEAR, *senior biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service,* with the assistance of JOSEPH LEITER and ADRIEN PERRAULT

Since the discovery of the carcinogenic activity of 1,2,5,6-dibenzanthracene and the isolation of 3,4-benzpyrene from coal tar, in Kennaway's laboratory in London, a great deal of attention has been focused on the individual hydrocarbons, while interest in the crude tars has declined. It is interesting to note that, whereas rabbits were employed in the first production of malignant tumors in experimental animals by coal tar, they are rather refractory even to large doses of the pure hydrocarbons. (For literature, see Shear (*1*).)

The possibility that other constituents of coal tar besides benzpyrene may play a part in the carcinogenic action of the crude tar has been considered in a preceding paper (*2*). It was found that certain fractions of creosote oil appeared to retard the production of skin tumors in mice by benzpyrene, whereas the basic fraction appeared to promote it. In those experiments, performed with female albino mice not of inbred stock, the benzene solutions employed in the painting contained 0.2 and 0.05 percent, respectively, of benzpyrene.

In this paper the results are recorded of further experiments with the basic fraction. Female strain A mice were painted with solutions containing 1 percent of the basic fraction together with 0.05 or 0.02 percent of benzpyrene. These low concentrations were employed with the expectation that the promoting effect of the basic fraction on tumor genesis might be manifested more clearly with minimal doses of carcinogen. In addition to the painting experiments, benzpyrene and the basic fraction dissolved in various concentrations in filtered lard were injected together subcutaneously. Similar injection experiments were carried out with other polycyclic carcinogens to ascertain whether the basic fraction would give any indication of an accelerating effect with other carcinogens besides benzpyrene.

These experiments are of an exploratory nature intended, among other things, to provide information as to which concentrations of carcinogen are most useful for further investigations of co-carcinogenic and anti-carcinogenic activity.

## MATERIALS AND METHODS

### PAINTING

One hundred female strain A mice [2] were divided into 5 groups of 20 each. They were painted on an average of 3 times weekly, according to the technique employed in the previous study. The 2 test solutions contained 1 percent of the basic fraction of creosote oil and in addition 0.05 or 0.02 percent of 3,4-benzpyrene. The 2 benzpyrene control solutions contained 0.05 and 0.02 percent, respectively, of this

[1] *This work was carried on at the U. S. Public Health Service, Office of Cancer Investigations, then located in the Wolcott Gibbs Memorial Laboratory, Harvard University.*

[2] *The mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.*

carcinogen, and no basic fraction. The other control solution contained 1 percent of the basic fraction, and no benzpyrene. Benzene was the solvent.

The 2 groups on which were used the test and control solutions containing 0.05 percent benzpyrene were painted 124 times over a period of 44 weeks (June 23, 1937–April 28, 1938). The other 3 groups were painted 145 times over a period of 51 weeks (June 23, 1937–June 17, 1938).

In comparing the results produced by the different solutions, the time of production of a tumor was arbitrarily defined as that required for the development of a growth to a diameter of about 2 mm. Only those neoplasms which grew progressively were included in the comparisons.

### SUBCUTANEOUS INJECTION

For the injection experiments, the carcinogens and the basic fraction were dissolved in lard which had been filtered at 38° C. The control solutions contained the hydrocarbon alone, or the basic fraction alone. A single injection of 0.2 to 0.3 cc. of the various solutions was given subcutaneously in the left axilla in all experiments, except in the few instances noted. As in the painting experiments, only strain A mice were employed. The empirical criteria employed in estimating the time of first appearance of a subcutaneous tumor have already been briefly discussed (*1*).

### SUBSTANCES EMPLOYED

The basic fraction of creosote oil was the same as that previously employed (*2*). The characteristics of the carcinogens obtained from our cooperating laboratories of organic chemistry have been described in the various papers [3] of Prof. L. F. Fieser and coworkers of Harvard University, and of Dr. M. S. Newman and coworkers of the Ohio State University. The commercial preparations were purified by Dr. J. L. Hartwell of this laboratory, until the melting points were equal to those specified by Fieser (*3*). The benzene used as solvent was of analytical reagent quality.

[3] *In the Journal of the American Chemical Society.*

## RESULTS

### PAINTING

The results of the painting experiments are summarized in figure 1. Curve I shows the rate of appearance of tumors in the control group painted with the solution containing 0.05 percent benzpyrene alone. This curve gives the total number of mice with induced skin tumors at the times shown. Curve II gives similar data for the 0.05-percent benzpyrene solution which contained in addition 1 percent of the basic fraction.

Both curves exhibit the same trends. The period of maximum tumor production was between 28 and 42 weeks. After this time, a few tumors developed slowly. Survival was excellent. Skin tumors were obtained in 19 of the 20 mice in the test group, and in 18 of the 20 mice in the control group. The difference between curves I and II indicates a tendency to earlier tumor production in the mice which were treated with the basic fraction; for example, at about the middle of the period of maximum tumor production (i. e., at 34 weeks), there were skin tumors in 13 of the test group as compared with 5 in the control group.

Tumors were produced more slowly with the lower concentration of benzpyrene (curves III and IV). At 44 weeks, when the experiment with 0.05 percent benzpyrene was nearly terminated, there were only two tumors in each of the two groups painted with 0.02 percent benzpyrene. At the lower concentration of benzpyrene both curves exhibited the same trends: Tumors appeared slowly at first and then more rapidly in both groups. As in the

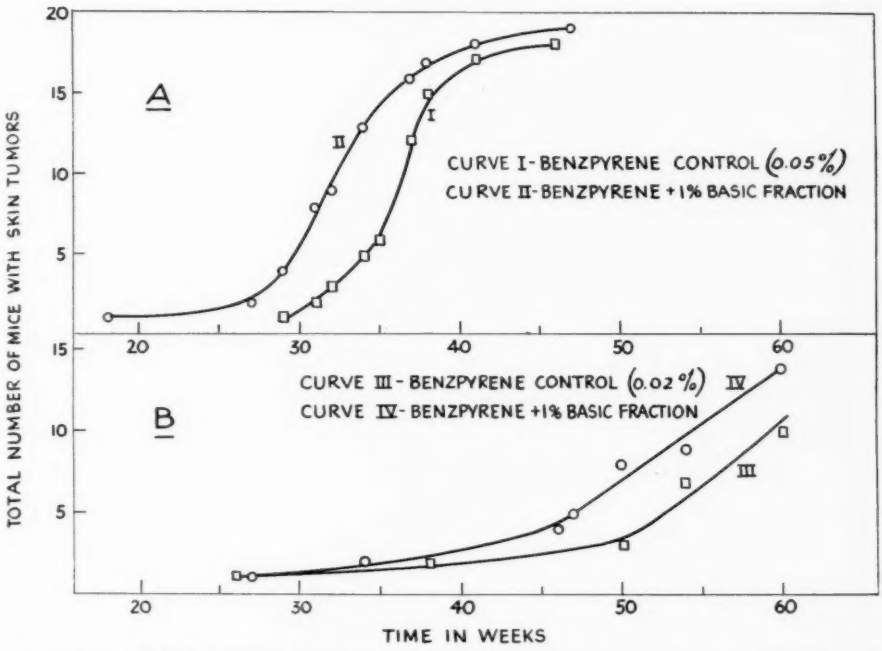



FIGURE 1.—Rate of skin-tumor production in strain A mice following painting with solutions of 3, 4-benzpyrene in benzene: *A*, 0.05-percent solution; *B*, 0.02-percent solution.

case of the higher benzpyrene concentration, there was a tendency for the tumors to appear earlier in the test group than in the control.

At 60 weeks, skin tumors had been produced with the test solution in 14 of the 20 test mice; the single survivor subsequently died without tumor. At the same time skin tumors had been produced in 10 of the 20 control mice; all 4 of the negative control animals which were alive at this time developed tumors subsequently (between 70 and 82 weeks).

No skin tumors developed in any of the 20 control mice painted with the benzpyrene-free solution containing 1 percent of the basic fraction. The survival rate was approximately the same as in the groups painted with the lower concentration of benzpyrene.

The results obtained in these painting experiments with strain A mice are in agreement with those previously obtained (*2*) with heterozygous albino mice painted with 0.05-percent benzpyrene and 1 percent of the basic fraction. In all three experiments, skin tumors appeared more rapidly when the solution contained the basic fraction in addition to benzpyrene.

### SUBCUTANEOUS INJECTION

The following experiments were next performed to see whether this apparent accelerating effect of the basic fraction on the production of skin tumors by the painting technique would also be observed following subcutaneous injection. Lard, filtered at 38° C., was the solvent in all cases.

### Experiment A—1 mg. Benzpyrene Plus Repeated Doses of 2 Percent of Basic Fraction

Each of 20 female mice received (July 14, 1937) an injection of 1 mg. benzpyrene in 0.2 cc. lard; 0.3 cc. of a 2-percent solution of the basic fraction in lard was injected at the same site 11 times in the course of the next 2 months. Each of 20 control females received the same amount of benzpyrene, and 11 injections of lard alone instead of the basic fraction solution. The results are summarized in table 1 which gives the total number of mice with induced tumors (plus signs), and the number of negative survivors (minus signs), after 4, 5, and 7 months, respectively.

Table 1.—*Effect of basic fraction of creosote oil on production of subcutaneous tumors by 3, 4-benzpyrene*

| Experiment | Dose injected | | Mice | | Total mice with tumors (+); negative [1] survivors (−) | | |
|---|---|---|---|---|---|---|---|
| | Benzpyrene | Basic fraction | Number | Sex | 4 months | 5 months | 7 months |
| | | Percent | | | Number | Number | Number |
| A | 1.0 mg. [2] | 2 [2] | 20 | F | 7+ | 15+ | 17+ |
| | | | | | 13− | 5− | 3− |
| | 1.0 mg. [2] | ..... | 20 | F | 11+ | 14+ | 15+ |
| | | | | | 8− | 5− | 4− |
| B | 2.5 mg. | 2 | 20 | M | 18+ | ..... | ..... |
| | | | | | 2− | ..... | ..... |
| | 2.5 mg. | ..... | 20 | M | 18+ | ..... | ..... |
| | | | | | 2− | ..... | ..... |
| | ..... | 2 [4] | 20 | M | 0+ | 0+ | 0+ |
| | | | | | 18− | 18− | 17− |
| C | 0.1 mg. [2] | 2 | 20 | M | 10+ | 17+ | 18+ |
| | | | | | 10− | 3− | 1− |
| | 0.1 mg. [2] | ..... | 20 | M | 1+ | 5+ | 6+ |
| | | | | | 19− | 15− | 14− |
| D | 1 percent [3] | 5 | 20 | F | 2+ | 4+ | 7+ |
| | | | | | 18− | 16− | 13− |
| | 1 percent [3] | ..... | 20 | F | 3+ | 4+ | 4+ |
| | | | | | 16− | 15− | 15− |
| | ..... | 5 | 10 | F | 0+ | 0+ | 0+ |
| | | | | | 10− | 10− | 10− |

[1] In these tables, mice other than those bearing induced tumors which grew progressively are classified as negative.
[2] Dose administered in 0.2–0.3 cc. of lard.
[3] 11 injections.
[4] 2 injections.
[5] Dose administered in cholesterol pellets.

It is clear from table 1 that no promoting effect was evident. If any effect was produced by the large amount of basic fraction, it was in the direction of retardation for, after 4 months, tumors had appeared in 7 of the test mice, as compared with 11 of the controls.

### Experiment B—0.5 mg. Benzpyrene Plus 2 Percent of Basic Fraction

When the amount of benzpyrene was cut in half, and only 1 injection of the basic fraction was given, there was still no difference in the rate of tumor production as contrasted with the benzpyrene control group. In this experiment, 20 male mice received (January 28, 1937) 0.25 cc. of a lard solution containing 0.2 percent benzpyrene and 2 percent of the basic fraction. The 20 male control mice received a similar amount of a lard solution containing only benzpyrene (0.25 cc. of a 0.2-percent solution contains 0.5 mg. benzpyrene). A second control group of 20 male mice received 1 injection of 0.25 cc. of a lard solution containing 2-percent solution of the basic fraction, but no benzpyrene.

The results, summarized in table 1, show that tumors developed very rapidly in the 0.5-mg. benzpyrene control group (males), in fact more rapidly than in the control experiment with 1.0 mg. benzpyrene (females). This result is consistent with the observations of Shear and Leiter, [4] on the more pronounced effect of borderline doses of benzpyrene in males than in females.[5] Another contributing factor, both in this and in subsequent experiments, may have been some variation in

[4] *Shear, M. J., and Leiter, J.: Unpublished experiments (1940).*

[5] *For example, in 27 experiments (790 mice) with various substances, in each of which 0.1 mg. benzpyrene in lard was injected subcutaneously in strain A mice of both sexes, tumors were obtained in 59 percent (221 tumors in 375 mice) of the males as contrasted with 41 percent (170 tumors in 415 mice) of the females.*

*In another similar series of 8 experiments (400 mice), in which 0.08 mg. benzpyrene in tricaprylin was employed, a similar contrast was obtained in each experiment. In this series, tumors were obtained in 56 percent (111 tumors in 200 mice) of the males, and in 37 percent (73 tumors in 200 mice) of the females.*

*For these 1,190 mice the tumor production was 58 percent in the males, and 40 percent in the females.*

the composition of the lard used in the different experiments; this subject also is being investigated further.

Addition of the basic fraction to the benzpyrene in this experiment produced no acceleration of tumor production; at the end of 4 months there were tumors in 18 of the 20 mice in both the test and the benzpyrene control groups.

The basic-fraction control group was entirely negative; no tumors developed in any of the mice in 14 months. In this group, the injection was repeated once after the first 6 months.

### Experiment C—0.1 mg. of Benzpyrene Plus 2 Percent of Basic Fraction

In the preceding experiments, tumors developed so rapidly in the control groups that it was conceivable that any promoting property of the basic fraction might have been obscured. Consquently, experiment B was repeated, but modified in the direction of a reduction in the amount of benzpyrene injected.

Both the control and test groups, each consisting of 20 male mice, received (May 26, 1937) 0.1 mg. of benzpyrene. The lard solution administered to the test mice contained, in addition, 2 percent of the basic fraction. The results are summarized in table 1.

With this low concentration of benzpyrene, tumors developed slowly in the control mice. After 7 months, only 6 of the mice bore tumors; all the remaining mice were alive and negative. In the eleventh month 1 more mouse developed a tumor; there were 10 mice alive and negative at this time. Tumors developed in 2 additional control animals by the time the experiment was terminated in the seventeenth month.

In striking contrast, tumors developed in 17 of the test mice in 5 months. Under these conditions the basic fraction apparently produced a pronounced promoting effect on tumor production.

### Experiment D—Cholesterol Pellets Containing Benzpryene and Basic Fraction

The effect of the basic fraction on the action of benzpyrene was also explored with the cholesterol pellet technique (*4*). A concentration of 1-percent benzpyrene in cholesterol was selected inasmuch as this appears to be a borderline dose (Shear and Ilfeld (*5*)).

Control pellets containing only benzpyrene were placed (September 13, 1937) subcutaneously in 20 female mice. Pellets containing 5 percent of the basic fraction in addition to the benzpyrene were inserted in 20 other females; and pellets containing only 5 percent of the basic fraction without benzpyrene were placed in another control group of 10 females. The results are summarized in table 1.

In the benzpyrene control group, as expected, tumors developed slowly and in only a small proportion of the mice. Of the 15 mice negative after 7 months, 10 survived for 15 months at which time the pellets were seen at autopsy in 5.

Of the 13 mice in the test group which were negative after 7 months, 4 survived for 15 months; the injected material was seen in 1 at this time. Tumors developed in 7 mice as contrasted with 4 in the benzpyrene control group.

No tumors developed in any of the 10 control mice which had received cholesterol pellets containing the basic fraction. When the surviving 5 mice were killed after 15 months, the pellets were seen in all. No gross tissue reaction was noted around the pellets in any of the negative mice at the termination of the experiment.

Although tumor production was 75 percent greater in the test group, the numbers of tumors obtained were too small to warrant considering the difference significant.

### Experiment E—0.1 mg. Dibenzanthracene Plus 2 Percent of Basic Fraction

The effect of the basic fraction on the action of several other hydrocarbons was also investigated. With 1,2,5,6-dibenzanthracene,[6] the 20 male mice in the control group each received (September 22, 1937) 0.1 mg. of the hydrocarbon in 0.2 cc. lard. The 20 males in the test group received 0.2 cc. of a solution containing 2 percent of the basic fraction in addition to 0.1 mg. of dibenzanthracene. The results are summarized in table 2.

Table 2.—*Effect of basic fraction of creosote oil on production of subcutaneous tumors by 1,2,5,6-dibenzanthracene and 20-methylcholanthrene*

[0.1 mg. of the carcinogen in each dose]

| Experiment | Dose injected [1] | | Mice | | Total mice with tumors (+). Negative [2] survivors (−) | | |
|---|---|---|---|---|---|---|---|
| | Carcinogen | Basic fraction | Number | Sex | 4 months | 6 months | 9 months |
| | | *Pct.* | | | *Number* | *Number* | *Number* |
| E.... | 1,2,5,6-dibenzanthracene. | 2 | 20 | M | 0+ | 6+ | 14+ |
| | | | | | 20− | 14− | 5− |
| | | ... | 20 | M | 0+ | 4+ | 7+ |
| | | | | | 19− | 15− | 12− |
| F.... | 20-methylcholanthrene. | 2 | 20 | M+F | 8+ | 18+ | ..... |
| | | | | | 12− | 2− | ..... |
| | | ... | 20 | M+F | 12+ | 18+ | ..... |
| | | | | | 8− | 2− | ..... |

[1] Each dose injected in 0.2 cc. of lard.
[2] See footnote 1 to table 1.

Tumors developed more rapidly in the mice of the test group than in the control mice (table 2). At the end of 9 months there were twice as many mice (14) in the test group that had developed tumors as compared with the controls (7); at this time there were 5 and 12 negative survivors in the respective groups. When the experiment was terminated after 17 months, tumors had developed in 14 mice in the test group and in 9 mice of the control group.

[6] *Hereafter referred to as dibenzanthracene.*

### Experiment F—0.1 mg. Methylcholanthrene Plus 2 Percent of Basic Fraction

An experiment similar to the preceding one was carried out with 20-methylcholanthrene [7] instead of dibenzanthracene. The chief difference was that 10 males and 10 females were employed in both the test and control groups instead of only males. The results are summarized in table 2.

Within each of the groups, tumors developed more rapidly in the males than in the females; this finding was consistent with similar results obtained in many other of our experiments in which low doses of carcinogens were employed.

As between the control and test groups, however, tumors developed somewhat more slowly in the mice that had received the basic fraction. This is seen on inspection of the results obtained at the end of 4 months. Subsequently, practically all of the mice developed tumors.

### Experiment G—0.02 mg. Methylcholanthrene Plus 5 Percent of Basic Fraction

Apparently 0.1 mg. of methylcholanthrene acted too rapidly because, of the 20 control mice, 12 and 16 developed tumors in 4 and 5 months, respectively. It was far more potent than 0.1 mg. benzpyrene (table 1, experiment C). In order to avoid obscuring by too high carcinogen concentration any promoting action of the basic fraction, the dose of methylcholanthrene was reduced to 0.02 mg. in experiment G (February 2, 1939). At the same time the concentration of the basic fraction was increased from 2 to 5 percent, in an attempt to enhance any promoting action. The results are summarized in table 3.

As in experiment F, tumors appeared more readily in the males of each group than in the females. No accelerating effect was noted in the test group; in fact the tumors developed somewhat more

[7] *Hereafter referred to as methylcholanthrene.*

TABLE 3.—*Effect of 5-percent basic fraction of creosote oil on production of subcutaneous tumors by borderline doses of carcinogens*

| Experiment | Dose[1] | Basic fraction | Mice: Number | Mice: Sex | Total mice with tumors (+). Negative[2] survivors (−): 4 months | 6 months | 9 months |
|---|---|---|---|---|---|---|---|
| | *Milligram* | *Percent* | | | *Number* | *Number* | *Number* |
| **METHYLCHOLANTHRENE** | | | | | | | |
| G | 0.02 | 5 | 20 | M+F | 1+ / 16− | 7+ / 8− | 9+ / 3− |
| | .02 | ..... | 20 | M+F | 6+ / 14− | 10+ / 8− | 10+ / 4− |
| **10-METHYL-1, 2-BENZANTHRACENE** | | | | | | | |
| H | 0.05 | 5 | 20 | M+F | 3+ / 15− | 5+ / 13− | 5+ / 10− |
| | .05 | ..... | 20 | M+F | 5+ / 15− | 6+ / 14− | 7+ / 9− |
| **9, 10-DIMETHYL-1, 2-BENZANTHRACENE** | | | | | | | |
| I | 0.05 | 5 | 20 | M+F | 6+ / 11− | 15+ / 1− | [3] 16+ / [3] 0− |
| | .05 | ..... | 20 | M+F | 6+ / 7− | 7+ / 6− | [3] 7+ / [3] 6− |
| **5-CONH$_2$-10-METHYL-1, 2-BENZANTHRACENE** | | | | | | | |
| J | 0.1 | 5 | 20 | M+F | 0+ / 20− | 1+ / 19− | 4+ / 16− |
| | .1 | ..... | 20 | M+F | 1+ / 15− | 3+ / 13− | 4+ / 9− |

[1] Injected in 0.2 cc. of lard.
[2] See footnote 1 of table 1.
[3] At 7 months, when the experiment terminated.

slowly than in the controls. At the end of 6 months, tumors had been obtained in 10 of the 20 control mice; none of the 8 negative survivors developed tumors by the time the experiment was terminated after 10 months. After 6 months, 7 of the test mice had tumors; of the 8 negative survivors, 2 developed tumors during the next 3 months.

This experiment shows that under these conditions 0.02 mg. methylcholanthrene is an appropriate dose for the study of promoting action, since the tumors developed slowly, and in only about half of the mice. The apparent retardation produced by the basic fraction may perhaps be ascribable to the high concentration (5 percent) employed. In subsequent experiments of Shear and Leiter [8] with various constituents of the basic fraction of creosote oil, it was found that high concentrations produced retardation of tumor development which was not noted with lower concentrations.

EXPERIMENT H—0.05 MG. 10-METHYL-1, 2-BENZANTHRACENE PLUS 5 PERCENT OF BASIC FRACTION

In an analogous experiment (February 21, 1939) with 10-methyl-1,2 benzanthracene (*6*), which had been shown (*1*) to be very potent in producing sarcomas in mice, it was found that 0.05 mg. of this hydrocarbon was an appropriate dose for study of promoting action. Tumors developed slowly in only a small proportion of the mice (table 3, experiment H). After 9 months, tumors had developed in only seven of the control mice; the nine negative survivors were still negative when the experiment was terminated in the eleventh month.

Addition of 5 percent of the basic fraction did not produce any pronounced effect. After 9 months tumors had been produced in only 5 mice; none of the 10 negative survivors developed tumors by the time the experiment was terminated in the eleventh month. There were too few tumors to give indication of any sex difference.

EXPERIMENT I—0.05 MG. 9, 10-DIMETHYL-1, 2-BENZANTRHACENE PLUS 5 PERCENT OF BASIC FRACTION

In an analogous experiment (February 21, 1939) with 9,10-dimethyl-1,2-benzanthracene (*7*), 0.05 mg. of the carcinogen was employed since 0.1 mg. (*1*) had produced sarcomas in 15 of 20 mice in 9 months.[9] Evidence of a promoting effect due to the basic fraction was obtained (table 3).

[8] *See footnote 4.*
[9] *Shear, M. J.: Unpublished experiment (1939).*

Although seven of the control mice were lost during the fourth month because of an epidemic infection of *Bacillus piliformis*, thus reducing the total number of control mice, the results still appear to have some significance since at the end of 9 months, there were no negative survivors in the test group as compared with six negatives in the control group. Both sexes developed tumors at about the same rate.

EXPERIMENT J—0.1 MG. 5-$CONH_2$-10-METHYL-1, 2-BENZANTHRACENE PLUS 5 PERCENT OF BASIC FRACTION

The rate of tumor production with this carcinogen (*9*) previously reported (*8*), of 6 tumors in 20 mice in 5 months, and of 15 tumors in 10 months, was for an experiment in which a dose of 2 mg. of the carcinogen was employed. Consequently, a dose of 0.1 mg. was selected for the new experiment in which 10 males and 10 females were used (February 28, 1939). The test solution contained, in addition to the carcinogen, 5 percent of the basic fraction.

Table 3 shows that 0.1 mg. is an appropriate dose of this compound for experiments on promoting action. However, no indication of a promoting action was shown by 5 percent of the basic fraction. When the experiment was terminated in the eleventh month, there were 11 negative survivors and 5 positive mice in the test group, as contrasted with 6 negative survivors and 5 positive mice in the control group. There were too few tumors to give indication of any sex difference.

## DISCUSSION

Several lines of evidence point to the existence of substances which, noncarcinogens themselves, promote the action of carcinogens. As mentioned previously, although coal tar produces skin tumors in rabbits and mice and although benzpyrene, a coal tar constituent, is a very effective carcinogen in skin-painting experiments with mice, the pure hydrocarbon is an unexpectedly weak carcinogen for rabbit skin. In earlier work (*2*), the basic fraction of creosote oil was found to be noncarcinogenic for mouse skin, and yet apparently accelerated skin-tumor production by a 0.05-percent solution of benzpyrene in benzene, in heterozygous albino mice.

In the experiments reported here, it was again found that the basic fraction did not produce skin tumors in mice, and similarly negative results were obtained for the subcutaneous tissues. Yet the addition of the basic fraction in 1-percent solution resulted in a more rapid production of skin tumors in strain A mice both with 0.05- and 0.02-percent solutions of benzpyrene.

In the earlier experiments no clear-cut evidence of a promoting action of the basic fraction was obtained when a 0.2-percent solution of benzpyrene (i. e., a relatively high one) was employed in the painting. In an analogous fashion, no evidence of promoting action was obtained when either 1.0 mg. or 0.5 mg. benzpyrene in lard solution was combined with the basic fraction. In these instances the rate of tumor production was already so rapid that the addition of the basic fraction produced no change.[10] However, when the amount of benzpyrene was reduced to 0.05 and 0.02 percent in the painting, and to 0.1 mg. in the injection experiment, the action of the carcinogen was not so great that it overwhelmed the effect of the basic fraction.

[10] *Similar considerations have been discussed by Baumann, Jacobi, and Rusch (10) in studying the effect of variation in diet on tumor production by chemical carcinogens. They stated that (10, p. 3) their results were complicated by several factors, among which was the possibility that "with both benzpyrene and methyl cholanthrene the rate of tumor production was so rapid that the modifying effect of other factors may have been overshadowed." This possibility had also been taken into account by Watson and Mellanby (11, 12) in their experiments with coal tar.*

From this point of view it is desirable to work out doses of the various carcinogens that would be suitable for studies of promoting action. A concentration (or dose) that produced tumors slowly in about half of the control animals would appear to be useful for such experiments.

For benzpyrene, 0.1 mg. gave the desired results. However, 0.1 mg. was too high for methylcholanthrene since it produced tumors in 18 of 20 mice in 6 months. When the dose was cut to 0.02 mg. of the hydrocarbon, the results in the control group were much better.

With dibenzanthracene, 0.1 mg. was satisfactory. With both the 10-methyl and 9,10-dimethyl derivatives of 1,2-benzanthracene, 0.05 mg. was satisfactory; with the 5-$CONH_2$-10-methyl derivative, 0.1 mg. was an appropriate dose.

In addition to the indications of a promoting action of the basic fraction given in the painting experiments of this and of the preceding study, some evidence was provided from the subcutaneous injection experiments. A striking promotion of tumor genesis was obtained with benzpyrene in experiment C. With dibenzanthracene (experiment E), with 9,10- dimethyl-1,2-benzanthracene (experiment I) and with cholesterol-benzpyrene pellets (experiment D), results consistent with a promoting effect were obtained.

There appear to be two factors which may account for the results of the negative experiments: (1) Too high doses of carcinogens may swamp the effect of any promoting agent present; and (2) too high doses of a promoting factor may actually retard tumor production.

Another source of irregularity is the lard used as solvent in the subcutaneous injection experiments. As mentioned in a preceding paper (*8*), variable results were obtained in duplicate experiments with a borderline dose of benzpyrene, when filtered lard of different batches was used as solvent. In subsequent experiments,[11] it was found that 0.1 mg. benzpyrene in the fraction of lard liquid at 38° C. produced tumors rapidly in a high proportion of the mice (in 36 of 50 mice in 4 months); the same dose of hydrocarbon in the fraction not liquid at 38° gave no tumors in 4 months in any of the 50 mice. The liquid fraction of lard also produced tumors more rapidly, when used as a vehicle for benzpyrene, than did tricaprylin. Because of these interesting properties of lard, this material is being studied further. It is emphasized that in each experiment the same batch of filtered lard was used for the test and the control solutions. Repetition of the experiment with the same specimens gave similar results. Control experiments indicate that the difference between the two lard fractions is due to their chemical composition and not to their physical condition.

The mechanisms by which fatty materials affect tumor genesis by chemical agents are still obscure although reports of such observations have appeared in the literature since 1930. In that year Watson and Mellanby obtained an increased production of skin tumors in mice with an extract of coal tar when mouse fat was applied to the skin or included in the diet. The work of Peacock and Beck (*13*), on the influence of the solvent (chicken fat, mouse fat, mouse lipoids, and lard) upon the production of subcutaneous tumors in several species of animals by hydrocarbons, was discussed in a previous paper (*8*). Baumann and Rusch (*14*) and Baumann, Jacobi, and Rusch (*10*) have found that mice on a high fat diet developed skin tumors, induced by ultraviolet light or by benzpyrene, more rapidly than mice on a control diet. Inasmuch as the fur of the fat-fed mice was quite greasy, Rusch, Baumann,

[11] *See footnote 4.*

and Kline (*15*) then investigated the local factors in the production of skin tumors by light. Their failure to obtain any acceleration of sarcoma production on the high fat diet may have been due, they stated, to the fact that the tumor production, by hydrocarbons, on the stock ration was already so rapid that the influence of the test diets may have been obscured.[12] Morton and Mider (*16, 17*) studied the effects of a petroleum ether extract of mouse carcasses on tumor formation in mice by benzpyrene. When the extract was painted on the skin about half an hour before benzpyrene was applied to the same area, an increased number of skin tumors was obtained; however, a solution of benzpyrene in this extract produced fewer skin tumors than did benzpyrene in benzene solution, and fewer subcutaneous tumors than did benzpyrene in sesame oil solution. With ethyl ether extracts of various mouse tissues, however, Shimkin and Andervont (*18*) obtained neither promotion nor inhibition of the genesis of sarcomas in mice with methylcholanthrene or dibenzanthracene.

## SUMMARY

1. The effect of the basic fraction of creosote oil on the carcinogenic action of 3, 4-benzpyrene and of related compounds was explored in 530 strain A mice.

In a prolonged skin-painting experiment, using female mice, and benzene as the solvent, tumors were obtained in 18 of 20 mice with a 0.05-percent solution of benzpyrene; the period of maximum tumor production was between 28 and 42 weeks.

2. With a 0.02-percent solution of benzpyrene, also in benzene, tumors were obtained in 14 of 20 mice in 82 weeks; the period of maximum tumor production was between 50 and 60 weeks.

With a 1-percent solution of the basic fraction, completely negative results were obtained.

When the solutions contained 1 percent of the basic fraction in addition to 0.05 or 0.02 percent benzpyrene, tumors developed more rapidly than in the control mice.

3. When single subcutaneous injections were made in mice of both sexes, filtered lard being used as the solvent, the rate of tumor production at relatively high concentrations of carcinogens was so rapid that any promoting effect of other factors may have been obscured. Concentrations which produced tumors slowly in about half of the control mice, were found to be as follows when filtered lard was the solvent:

| Carcinogen: | *Mg.* |
|---|---|
| 3, 4-benzpyrene | 0.1 |
| 1, 2, 5, 6-dibenzanthracene | .1 |
| 20-methylcholanthrene | .02 |
| 10-methyl-1, 2-benzanthracene | .05 |
| 9, 10-dimethyl-1, 2-benzanthracene | .05 |
| 5-$CONH_2$-10-methyl-1, 2-benzanthracene | .1 |

Evidence of a promoting effect of the basic fraction on the production of subcutaneous tumors was obtained in some of the experiments.

The absence of a promoting effect in the experiments with single subcutaneous injections, in which the concentration of carcinogen was regarded as satisfactory, is tentatively ascribed to too high a concentration of the basic fraction.

[12] *See footnote 10.*

## REFERENCES


(*1*) SHEAR, M. J.: Studies in carcinogenesis. V. Methyl derivatives of 1:2-benzanthracene. Am. J. Cancer, **33:** 499–537 (1938).

(*2*) CABOT, S., SHEAR, N., and SHEAR, M. J.: Studies in carcinogenesis. XI. Development of skin tumors in mice painted with 3:4-benzpyrene and creosote oil fractions. Am. J. Path., **16:** 301–312 (1940).

(*3*) FIESER, L. F.: Carcinogenic activity, structure, and chemical reactivity of polynuclear aromatic hydrocarbons. Am. J. Cancer, **34:** 37–124 (1938).

(*4*) SHEAR, M. J.: Studies in carcinogenesis. I. The production of tumors in mice with hydrocarbons. Am. J. Cancer, **26:** 322–332 (1936).

(*5*) SHEAR, M. J., and ILFELD, F. W.: Studies in carcinogenesis. IX. Hydrocarbon—cholesterol pellets in strain D mice. Am. J. Path., **16:** 287–293 (1940).

(*6*) FIESER, L. F., and NEWMAN, M. S.: The synthesis of 1,2-benzanthracene derivatives related to cholanthrene. J. Am. Chem. Soc., **58:** 2376–2382 (1936).

(*7*) NEWMAN, M. S.: The synthesis of 9,10-dimethyl-1,2-benzanthracene. J. Am. Chem. Soc., **60:** 1141–1142 (1938).

(*8*) SHEAR, M. J.: Studies in carcinogenesis. VII. Compounds related to 3:4-benzpyrene. Am. J. Cancer, **36:** 211–228 (1939).

(*9*) NEWMAN, M. S.: The synthesis of 5-chloro-10-methyl-1,2-benzanthracene and related compounds. J. Am. Chem. Soc., **60:** 1368–1370 (1938).

(*10*) BAUMANN, C. A., JACOBI, H. P., and RUSCH, H. P.: The effect of diet on experimental tumor production. Am. J. Hyg., **30** (Sect. A): 1–6 (1939).

(*11*) WATSON, A. F., and MELLANBY, E.: Tar cancer in mice; technique of comparative experiment. Brit. J. Exper. Path., **11:** 267–281 (1930).

(*12*) WATSON, A. F., and MELLANBY E.: Tar cancer in mice; condition of skin when modified by external treatment or diet, as factor in influencing cancerous reaction. Brit. J. Exper. Path., **11:** 311–322 (1930).

(*13*) PEACOCK, P. R., and BECK, S.: Rate of absorption of carcinogens and local tissue reaction as factors influencing carcinogenesis. Brit. J. Exper. Path., **19:** 315–319 (1938).

(*14*) BAUMANN, C. A., and RUSCH, H. P.: Effect of diet on tumors induced by ultraviolet light. Am. J. Cancer, **35:** 213–221 (1939).

(*15*) RUSCH, H. P., BAUMANN, C. A., and KLINE, B. E.: Effect of local applications on development of ultraviolet tumors. Proc. Soc. Exper. Biol. & Med., **42:** 508–512 (1939).

(*16*) MORTON, J. J., and MIDER, G. B.: Effect of petroleum ether extract of mouse carcasses as solvent in production of sarcoma. Proc. Soc. Exper. Biol. & Med., **41:** 357–360 (1939).

(*17*) MORTON, J. J., and MIDER, G. B.: Effect of petroleum ether extract of mouse carcasses on skin tumor production in C57 black mice. Pub. Health Rep., **55:** (1940).

(*18*) SHIMKIN, M. B., and ANDERVONT, H. B.: Factors influencing carcinogenesis with methylcholanthrene. III. The effect of solvents. Pub. Health Rep., **55:** 537–545 (1940).

# COMPARATIVE CARCINOGENICITY OF THREE CARCINOGENIC HYDROCARBONS

By MICHAEL B. SHIMKIN, *assistant surgeon*, and HOWARD B. ANDERVONT, *senior biologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

The determination of the relative carcinogenicity of structurally related polycyclic aromatic hydrocarbons, as well as the information whether any particular compound is carcinogenic or not, is essential for the important investigations of the relation of the molecular structure of these chemicals to their carcinogenic activity.

The carcinogenic potency of a series of hydrocarbons, prepared by Fieser and his coworkers (*1*), has been determined by Shear (*2–4*) in this laboratory. The method employed was the single subcutaneous injection into strain A mice of about 5 to 10 mg. of the crystalline compound moistened with glycerin. This procedure gives the qualitative answer to whether the particular chemical is able to initiate neoplastic growth at the site of injection or not, but only a rough approximation of its carcinogenic activity as compared with related chemicals.

For more exact comparisons of relative carcinogenic activities of different hydrocarbons it is desirable to inject various doses of the compounds, up to and including the dose above which no increase in the incidence and no decrease in the latent period can be obtained, in one standard physical state and medium, into standard experimental animals which are as free of variables as possible.

The following investigations, designed to establish such procedure and based upon previous studies (*5–10*), were undertaken at the instigation of Prof. L. F. Fieser. Concurrently, an experiment was performed to ascertain the value of nonhomozygous mice in this type of research.

## EXPERIMENTAL PROCEDURE

The animals used were male mice of strain $C_3H$, obtained from the Roscoe B. Jackson Memorial Laboratory, and male and female white mice of undetermined genetic constitution obtained from a local dealer. All were kept under identical environmental and dietetic conditions and were from 2 to 3 months of age at the time of injection. The diet consisted of Purina dog-chow pellets and an unlimited supply of water.

The carcinogenic agents employed were 20-methylcholanthrene (melting point 179.8°–180.4° C., corr.), 3,4-benzpyrene (melting point 178.6°–179.0° C., corr.), and 1,2,5,6-dibenzanthracene (melting point 266.0°–266.8° C., corr.).[1] The solvent used was tricaprylin,[2] which was determined previously to be an excellent vehicle for methylcholanthrene (*10*); the glyceride was slightly yellow in color and had a freezing point of 8.6°–8.8° C. (corr.).

The various doses of the hydrocarbons were dissolved in 0.2 cc. of the solvent, and administered to mice in a single subcutaneous injection into the right axilla. The animals were examined weekly. As soon as a hard mass which could not be dissipated by pressure was palpable, the mouse was marked and permitted to live

[1] *Throughout the paper, the compounds will be referred to simply as methylcholanthrene, benzpyrene, and dibenzanthracene.*

[2] *Procured from Dr. H. H. Young, of the Swift & Co. Research Laboratory, through Professor Fieser.*

until a growing tumor was diagnosed grossly, when the animal was killed and autopsied.

The appearance of the tumors was recorded weekly, according to the time a hard mass was palpable. Mice dying of extraneous causes before tumors began to appear in the particular series were subtracted from the totals of the animals injected.

The averages of latent periods were computed by multiplying the number of tumors appearing each week by the time in weeks after injection, and dividing the sum by the total number of tumors. The 50-percent-average latent periods were calculated by the same method, but only the first 50 percent of the animals which developed tumors in each group were considered. The carcinogenic indices, a value suggested by Iball (*11*), were determined by the formula $P/T \times 100$, in which $P$ is the percent of mice developing tumors and $T$ the average latent period of the whole group in days.

## EXPERIMENT 1

Two hundred and forty $C_3H$ male mice were divided into groups of 20, and injected with 0.25, 0.5, 1.0, or 2.0 mg. of methylcholanthrene, benzpyrene, or dibenzanthracene in 0.2 cc. tricaprylin. Another 18 mice received 3.0 mg. dibenzanthracene in 0.2 cc. tricaprylin.

Solution was effected by heating. The preparations of 2.0 mg. methylcholanthrene or dibenzanthracene in 0.2 cc. tricaprylin remained in solution at 40° C., but partially precipitated on cooling to 20° C.; 3.0 mg. dibenzanthracene did not remain in solution at 40° C. and was injected as a suspension. Thus, except for the 3.0 mg. dibenzanthracene, all the concentrations were in a dissolved state at the body temperature of the mouse.

Seven mice died of intercurrent infection before the termination of the experiment. All had received dibenzanthracene. The mortality is not attributed to the toxicity of the compound but to the long latent period in these groups. Two animals, one injected with 2.0 mg. and the other with 3.0 mg. dibenzanthracene developed slight ulceration at the site of injection. No external evidence of irritation was present in the remainder of the animals.

The injected material could be felt for many weeks after the administration; in many mice, some encapsulation was present. About a week before tumors began to appear in the mice, there was definite thickening of the subcutaneous tissue at the site of injection. Especially with the use of methylcholanthrene, a soft, doughy mass could be felt over a wide area about the injection site a week before definite hard tumors arose.

The latent periods of the tumors are recorded in table 1. From the data it is evident that methylcholanthrene is the most carcinogenic compound of the three tested, in all doses, and that benzpyrene and dibenzanthracene follow in the order named. This is in agreement with previous studies (*11*, *12*) of the agents. The results also permit the following conclusions: (1) There is a minimal latent period for each of the three hydrocarbons; when the hydrocarbon is dissolved in 0.2 cc. tricaprylin and injected subcutaneously into $C_3H$ mice, it is 6 weeks for methylcholanthrene, 10 weeks for benzpyrene, and 12–13 weeks for dibenzanthracene; (2) the spread of the latent period is shortest with methylcholanthrene, 9 weeks, and lengthens out with benzpyrene, 15 weeks, and with dibenzanthracene, 25 weeks; (3) the average or 50-percent latent periods are least for methylcholanthrene and greatest for dibenzanthracene; and (4) the maximum effective dose, i. e., the dose above which no increase in the inci-

TABLE 1.—*Induction of subcutaneous tumors in $C_3H$ male mice with single subcutaneous injection of carcinogenic hydrocarbons*

| Hydrocarbon | Dose | Mice | Number of tumors per week and time (in weeks) | | | | | | | | | | | | | | | | | | | | | | | | | Tumors | Average latent time | 50-percent latent time | Carcinogenic index |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32+ | | | | |
| | *Mg.* | *No.* | | | | | | | | | | | | | | | | | | | | | | | | | | | | *No.* | *Weeks* | *Weeks* | |
| Methylcholanthrene | 0.25 | 20 | .. | .. | 3 | 5 | 4 | 5 | 1 | 1 | 1 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 20 | 10.1 | 8.9 | 140 |
| | .5 | 20 | 1 | 3 | 3 | 1 | 5 | 5 | 1 | 1 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 20 | 9.5 | 8.0 | 150 |
| | 1.0 | 20 | 1 | 3 | 3 | 5 | 3 | 3 | 2 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 20 | 9.1 | 7.8 | 155 |
| | 2.0 | 20 | .. | 1 | 4 | 3 | 2 | 6 | 3 | .. | 1 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 20 | 10.1 | 8.6 | 140 |
| Benzpyrene | .25 | 20 | .. | .. | .. | .. | .. | 2 | 1 | 1 | 4 | 2 | 2 | 2 | 2 | 1 | .. | .. | .. | .. | 1 | .. | .. | .. | .. | .. | .. | .. | .. | 18 | 15.4 | 13.3 | 90 |
| | .5 | 20 | .. | .. | .. | .. | 1 | 5 | 1 | 3 | 2 | 2 | 1 | 1 | 2 | 1 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 19 | 13.8 | 11.6 | 100 |
| | 1.0 | 20 | .. | .. | .. | .. | 1 | 7 | 3 | 5 | 2 | .. | 1 | 1 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 20 | 12.5 | 11.1 | 115 |
| | 2.0 | 20 | .. | .. | .. | .. | .. | 9 | 2 | 3 | 2 | 3 | .. | .. | .. | .. | 1 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 20 | 12.1 | 11.1 | 120 |
| Dibenzanthracene | .25 | 19 | .. | .. | .. | .. | .. | .. | .. | .. | .. | 1 | .. | 2 | .. | .. | 3 | 1 | 2 | 1 | 1 | .. | 2 | .. | 1 | 1 | .. | 1 | 2 | 17 | 24.6 | 20.6 | 50 |
| | .5 | 20 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 2 | 1 | .. | .. | 2 | 1 | 1 | 1 | 3 | 2 | 1 | .. | 2 | 1 | .. | .. | 18 | 23.4 | 20.3 | 55 |
| | 1.0 | 20 | .. | .. | .. | .. | .. | .. | .. | 1 | 1 | 1 | .. | .. | 2 | .. | 2 | 1 | 2 | 2 | 1 | 2 | 1 | 2 | .. | 1 | .. | .. | .. | 19 | 21.7 | 18.3 | 60 |
| | 2.0 | 18 | .. | .. | .. | .. | .. | .. | 2 | 2 | 3 | 1 | .. | 2 | 2 | 2 | 1 | .. | .. | .. | 1 | .. | .. | .. | 1 | .. | .. | .. | .. | 17 | 17.7 | 14.8 | 75 |
| | 3.0 | 16 | .. | .. | .. | .. | .. | .. | 1 | 1 | 3 | 2 | 1 | 2 | 1 | .. | 2 | 2 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 15 | 15.9 | 14.1 | 85 |

dence and no decrease in the latent period were obtained, is smallest for methylcholanthrene, 0.25 mg.; next for benzpyrene, 1 mg.; and greatest for dibenzanthracene, 2.0 mg. The experiment substantiates Fieser's (*1*) hypothesis that rapidity of action goes hand in hand with the ability to exert a maximum response at low dosage.

### EXPERIMENT 2

The review of the subject (*1*) indicates that there is apparently no difference in the results obtained with multiple as compared with single subcutaneous injections of carcinogenic hydrocarbons. The former method, however, is open to several objections: (1) There are no direct data that carcinogenesis with multiple injections is identical with that obtained with single injections; (2) with rapidly acting agents such as methylcholanthrene, where tumors are expected as soon as 6 weeks after administration, it is questionable whether a repeated injection on the fourth or later week would have time to influence the process; thus the actual effective dose will have been lower than indicated; and (3) the interpretation of the latent period is difficult, since if the time is determined from the first injection, only that portion of the total dose given at the original administration will have exerted its action during the period, whereas later doses will have acted for a shorter period. Thus the method is not directly comparable, a priori, with the single injection in which the total dose will have had the opportunity to act during the total latent period.

The following experiment was therefore performed. Thirty-two strain $C_3H$ male mice, of the same age as those used previously, were injected in the right axilla four times at weekly intervals with 0.125 mg. dibenzanthracene in 0.05 cc. tricaprylin. Thus in 4 weeks the mice received 0.5 mg. of the hydrocarbon and are comparable with the 20 mice which were given a single injection of 0.5 mg. dibenzanthracene.

Table 2 shows that the incidence of tumors, the minimal latent periods, the spread of induction times, and the average latent periods were remarkably close in the two groups. Multiple injections, therefore, have the same effect as single injections of dibenzanthracene. This is interesting, because it indicates that probably

TABLE 2.—*Effect of repeated versus single injections of dibenzanthracene on induction of subcutaneous tumors*

| Injections | Dose | Mice | Number of tumors per week and time (in weeks) | | | | | | | | | | | | | | | | | | | | Tumors | Average latent time |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | |
| | *Mg.* | *No.* | | | | | | | | | | | | | | | | | | | | | | *Number* | *Weeks* |
| Single | 0.5 | 20 | ... | 1 | ... | 2 | 1 | ... | ... | 2 | 1 | 1 | 1 | 3 | 2 | 1 | ... | 2 | ... | 1 | ... | ... | ... | 18 | 23.4 |
| Repeated | [1].5 | 32 | 1 | 1 | 2 | ... | 3 | 1 | 2 | ... | 1 | 2 | 2 | 4 | 1 | 3 | ... | 1 | 2 | 1 | ... | 2 | ... | 29 | 24.2 |

[1] Administered in 4 weekly doses of 0.125 mg.

not all of the injected hydrocarbon is at once effective in producing tumors at the site of injection, and that repeated stimulation does not accelerate the process.

It is also apparent that since single injections are as efficacious as multiple ones, the former technique is more desirable from the standpoint of ease and simplicity.

EXPERIMENT 3

It has been the policy of this laboratory to use only inbred strains of mice in experiments with carcinogenic hydrocarbons (*2–10*). Since the different strains of mice differ widely in their susceptibility to the agents (*7*), as determined by the incidence and the latent periods of sarcogenesis, it is to be expected that results with nonhomozygous animals would vary to a greater extent than with inbred mice.

In order to ascertain the advantages and disadvantages of the use of stock mice in these investigations, the following experiment was performed: From 15 to 20 white market mice of both sexes were injected with 0.25, 0.5, 1.0, or 2.0 mg. of methylcholanthrene, benzpyrene, or dibenzanthracene. There was a high mortality among the animals, owing to pneumonia, and about 10 of the mice had tapeworm of the liver. The significant totals in each group and the induction of tumors are presented in table 3. There was ulceration at the site of injection in 15 to 20 percent of the mice injected with 1.0 mg. or more of the hydrocarbons. The experiment was terminated at 40 weeks, which probably accounts for the low incidence of tumors obtained with dibenzanthracene.

Despite the small number of animals in each group, the same relative results were obtained with the stock mice as with the inbred mice, i. e., methylcholanthrene produced the most tumors in the shortest period of time, with benzpyrene and dibenzanthracene next in order. The

TABLE 3.—*Induction of subcutaneous tumors in stock mice with single subcutaneous injection of carcinogenic hydrocarbons*

| Hydrocarbon | Dose | Mice | Number of tumors per week and time (in weeks) | | | | | | | | | | | | | | | | | | | | | | | Tumors | Average latent time |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34+ | |
| | *Mg.* | *No.* | | | | | | | | | | | | | | | | | | | | | | | | | | | *No.* | *Wks.* |
| Methylcholanthrene | 0.25 | 11 | ... | ... | ... | ... | ... | 1 | 1 | 1 | ... | ... | 1 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | 1 | ... | 5 | 18.8 |
| | .5 | 17 | ... | 1 | 3 | 2 | 1 | 1 | 3 | 1 | ... | ... | 1 | ... | ... | ... | 1 | 1 | ... | 1 | ... | ... | ... | ... | ... | ... | ... | ... | 16 | 15.4 |
| | 1.0 | 11 | ... | ... | 1 | ... | ... | 1 | 1 | 1 | ... | 1 | ... | ... | 1 | ... | ... | ... | ... | 2 | ... | ... | ... | ... | ... | ... | ... | 1 | 9 | 20.4 |
| | 2.0 | 17 | 3 | 2 | ... | 1 | ... | 3 | 2 | 1 | 1 | ... | ... | 1 | ... | ... | 1 | ... | ... | ... | ... | 1 | ... | ... | ... | ... | ... | ... | 16 | 14.7 |
| Benzpyrene | .25 | 12 | ... | ... | ... | ... | ... | 2 | ... | ... | ... | ... | ... | ... | ... | 1 | 1 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | 4 | 18.2 |
| | .5 | 18 | ... | ... | ... | ... | ... | 1 | ... | 1 | ... | 2 | 1 | 3 | ... | ... | ... | ... | ... | 1 | 1 | ... | ... | ... | ... | ... | ... | ... | 10 | 19.8 |
| | 1.0 | 12 | ... | ... | ... | ... | ... | ... | 1 | ... | ... | 2 | ... | 2 | ... | ... | ... | ... | ... | ... | 1 | ... | 1 | ... | ... | ... | 1 | ... | 8 | 22.5 |
| | 2.0 | 15 | ... | ... | ... | ... | ... | 3 | ... | ... | ... | 2 | ... | 1 | ... | 1 | ... | ... | ... | ... | 1 | 1 | ... | ... | ... | ... | ... | ... | 9 | 19.1 |
| Dibenzanthracene | .25 | 15 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | 1 | ... | 1 | ... | ... | ... | ... | ... | ... | ... | 2 | 26.0 |
| | .5 | 16 | ... | ... | ... | ... | ... | ... | ... | ... | 1 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | 1 | ... | ... | ... | ... | ... | 2 | 22.5 |
| | 1.0 | 20 | ... | ... | ... | ... | ... | ... | ... | ... | ... | 2 | ... | 1 | ... | ... | ... | ... | 1 | ... | ... | ... | ... | ... | ... | ... | ... | 2 | 6 | 26.7 |
| | 2.0 | 16 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | 1 | ... | ... | ... | ... | ... | ... | ... | ... | 1 | ... | 1 | 4 | 30.1 |

results are not so closely grouped and not so clear-cut as with the $C_3H$ mice, and no conclusions concerning the spread of the latent period or the maximum effective dose can be reached, probably because of the greater resistance of the mice, their lack of homozygosis, and the small number of animals.

The stock mice of nonhomozygous constitution, therefore, are adequate for the gross determination and evaluation of carcinogenic hydrocarbons (*13*), although they are not so desirable for more quantitative lines of research.

## DISCUSSION

No attempt was made to keep individual records of the tumors. The masses were fixed by groups in Tellyesniczky's fluid and single sections were stained with haematoxylin and eosin. Histologic examination [3] was done to ascertain the error of the method employed.

The tumors were divided into two groups, since no gross or microscopic differences were apparent in the tumors induced by the three hydrocarbons, or by the different dosages of the compounds (1) tumors induced in the $C_3H$ mice and (2) tumors in the stock mice. In the former, the animals were killed as soon as a growing mass was palpable, usually 1 week after the detection of a hard mass at the site of injection. In the latter, the mice were permitted to live slightly longer, about 1 more week, so that the masses on the average were larger than those in the $C_3H$ mice.

Of the 260 tumors in $C_3H$ mice examined, 241 were histologically malignant. Of these, 237 were spindle-cell sarcomas, and 4 were squamous-cell carcinomas. Seventeen mice did not have definite neoplasms. In 16 of these 17, the lesion at the site of injection showed inflammatory and hyperplastic tissue resembling that which antedates sarcoma, and 1 mouse had a papilloma. Actual errors in diagnosis were made in 2 mice; one had a chronic inflammatory lesion with hemorrhage, and in one a hyperplastic lymph node was mistaken for a tumor.

Thus, in mice killed as soon as a gross diagnosis of sarcoma was made, there was at the most a 7-percent (19 out of 260) error as checked by histologic examination.

That this error can be reduced by permitting the animals to live slightly longer is evident from the results with stock mice. Of the 76 masses examined, 72 were sarcomas and 4 were carcinomas. In 76 mice, therefore, the error in diagnosis was 0 percent.

## SUMMARY

A uniform experimental procedure for the determination of relative potencies of carcinogenic and related polycyclic aromatic hydrocarbons is presented. The chemically pure compound was dissolved in an agent of known chemical composition, such as tricaprylin, and used in a range of doses, including the maximum effective dose. The solution was administered as a single subcutaneous injection into male mice of $C_3H$ strain, 2 to 3 months of age. The animals were maintained on the same dietary and environmental conditions. Tumors were detected by weekly palpatory examination, and the animals killed as soon as a growing hard mass at the site of injection was present. The gross diagnosis was substantiated by histologic study.

The error of the gross diagnosis can be reduced to less than 1 percent by permitting the tumors to grow for 2 to 3 weeks after the original appearance. The data

[3] *We are indebted to Drs. Harold L. Stewart and G. Burroughs Mider for assistance in histologic examination.*

can be interpreted in several ways, such as by the minimal, average, or 50-percent latent time, or by the carcinogenic index. The results depend upon the number of animals used. At least 20 mice should be injected with each dose of the compound.

The experiment with methylcholanthrene, benzpyrene, and dibenzanthracene, planned according to the above, showed that quantitative data can be obtained by the technique. It was demonstrated that the relative potency of the three hydrocarbons is in the order named.

Single subcutaneous injections were as efficacious as repeated injections in eliciting sarcomas with dibenzanthracene.

Inbred strains of mice are valuable in quantitative investigations with the carcinogenic hydrocarbons. Stock mice of nonhomozygous constitution are adequate only for gross evaluations of the compounds.

Acknowledgment is made of the technical assistance of Mr. Walter F. Gately.

## REFERENCES

(*1*) Fieser, L. F.: Carcinogenic activity, structure, and chemical reactivity of polynuclear aromatic hydrocarbons. Am. J. Cancer, **34:** 37 (1938).

(*2*) Shear, M. J.: Studies in carcinogenesis. III. Isomers of cholanthrene and methylcholanthrene. Am. J. Cancer, **28:** 334 (1936).

(*3*) ——— Studies in carcinogenesis. V. Methyl derivatives of 1:2-benzanthracene. Am. J. Cancer, **33:** 499 (1938).

(*4*) ——— Studies in carcinogenesis. VII. Compounds related to 3:4-benzpyrene. Am. J. Cancer, **36:** 211 (1939).

(*5*) Andervont, H. B., and Lorenz, E.: Dibenzanthracene tumors in mice; production of subcutaneous, pulmonary, and liver tumors by serum dispersions and lard solutions. Pub. Health Rep., **52:** 637 (1937).

(*6*) Andervont, H. B., and Lorenz, E.: Dibenzanthracene tumors in mice; production of subcutaneous and pulmonary tumors by 1:2:5:6-dibenzanthracene adsorbed on charcoal. Pub. Health Rep., **52:** 1931 (1937).

(*7*) Andervont, H. B.: Susceptibility of mice to spontaneous, induced and transplantable tumors; comparative study of 8 strains. Pub. Health Rep., **53:** 1647 (1938).

(*8*) Shimkin, M. B.: Factors influencing carcinogenesis with methylcholanthrene. I. Effect of age. Pub. Health Rep., **54:** 1388 (1939).

(*9*) Shimkin, M. B., and Andervont, H. B.: Factors influencing carcinogenesis with methylcholanthrene. II. Lack of effect of foster nursing. Pub. Health Rep., **54:** 2080 (1939).

(*10*) Shimkin, M. B., and Andervont, H. B.: Factors influencing carcinogenesis with methylcholanthrene. III. Effect of solvents. Pub. Health Rep., **55:** 537 (1940).

(*11*) Iball, J.: The relative potency of carcinogenic compounds. Am. J. Cancer, **35:** 188 (1939).

(*12*) Twort, J. M., and Twort, C. C.: Comparative activity of some carcinogenic hydrocarbons. Am. J. Cancer, **35:** 80 (1939).

(*13*) Cook, J. W., Haslewood, G. A. D., Hewett, C. L., Hieger, I., Kennaway, E. L., and Mayneord, W. V.: Chemical compounds as carcinogenic agents. Am. J. Cancer, **29:** 219 (1937).

# THE EFFECT OF VARIATION IN OXYGEN TENSION AND SULFHYDRYL CONCENTRATION ON NUCLEAR GROWTH AND FISSION IN AMOEBA PROTEUS

By H. W. CHALKLEY, *physiologist*, and CARL VOEGTLIN, *Chief, National Cancer Institute, National Institute of Health, United States Public Health Service*

This paper deals with certain fundamental biochemical aspects of the division process of normal cells. The ultimate understanding of the apparently unlimited proliferation of cancer cells must come from a comparison of the various factors which control normal as well as cancer cells.

In previous work, Chalkley and Voegtlin (*1*) concluded that the stimulative effect of glutathione and the inhibitory effect of copper on nuclear fission in *Amoeba proteus* were correlated with the effects of these reagents upon nuclear growth measured as change in nuclear volume. We also pointed out the possibility that these effects might be linked with the action of an enzyme or enzymes of the cathepsin type. Later it was shown that there exists in the resting nucleus of the amoeba a relatively high concentration of SH and that this concentration undergoes a cyclic variation during interkinesis and fission (*2*).

Voegtlin, Maver, and Johnson (*3*), working with cathepsin *in vitro*, found that this enzyme was activated by SH and inhibited by traces of copper salts. They also found that under anaerobic conditions the lytic activity was enhanced, but that in a strongly oxygenated solution of concentrated split products protein synthesis occurs, as shown by a marked increase in trichloracetic acid precipitable protein. This raised the question as to whether the growth of the nucleus necessarily involving protein synthesis might not be similarly affected, i. e., depressed by anaerobic conditions and stimulated by oxygenation, assuming that this growth was controlled in part at least by an enzyme of this type. We therefore subjected young amoebae of known age, adult amoebae, and amoebae undergoing fission to increased and decreased oxygen tension without and with addition of SH glutathione, and also to NaCN. We ascertained the effect of these conditions on the percentage of fission occurring in a given period, on the growth of the nucleus, and on the velocity of mitosis.

Since, under survival conditions, which obtained in all experiments, the cell volume always decreases irrespective of the change in nuclear volume, provided that toxic conditions do not supervene (*1*), it was deemed unnecessary to keep accurate records of such changes in cell volume as occurred. A few rough measurements were made at various times, especially when marked increase in the nuclear volume was noted. In all instances the results conformed to our previous experience in that there was a decrease in cell volume during the experiment.

The results obtained showed that (1) there occurs a variation in the nuclear growth correlated with both the oxygen tension and SH concentration of the cell environment, that (2) this tension and concentration do not affect the process of fission per se, and (3) that the percentage of fission occurring in a given period is

closely correlated with the rate of nuclear growth in every set of conditions employed.

## MATERIAL AND METHODS

The amoebae used were from a clone culture kept under standard conditions in this laboratory since 1928. All glassware was of Pyrex glass and great care was taken in its cleaning. All solutions were made up in glass-distilled water. The cultures were kept and all experiments were performed in a constant temperature room at 23° to 24° C. The glutathione used was prepared by Dr. J. M. Johnson of this laboratory, and was especially purified to remove copper. The salts used were of reagent grade. Ordinary tank nitrogen and oxygen approximately 99.5 percent pure were used since their absolute freedom from traces of other atmospheric gases was not essential. The saline medium used throughout in the solutions was made up as follows: 0.1 gm. NaCl, 0.004 gm. KCl, 0.006 gm. $CaCl_2$, plus distilled water to make 1,000 cc. To each 1,000 cc. was added 3 to 5 cc. of phosphate buffer at pH 7.0. The pH of the culture media and solutions used was in all cases between 6.8 and 7.0. All solutions were made up freshly before each experiment.

In experiments with cells in fission, amoebae were removed from culture as soon as they entered prophase, washed, and placed singly in small Pyrex dishes containing from 0.2 to 0.6 cc. of solution, and then subjected to the requisite treatment. (For details of selection, see Chalkley and Daniel (*4*).)

In experiments upon young cells when measurement of the nuclear volume and growth was to be made, cells at any state of fission were selected from culture, placed in small dishes in standard saline, and allowed to complete their fission. The daughter cells were placed in separate dishes with a little [1] food and left 24 hours. After this each cell was placed in a hanging drop preparation, transferred to the stage of a compound microscope, and the volume of the nucleus was measured as follows: By means of a camera lucida, outline drawings were made of the nucleus as seen "en plan" and as seen from the edge. At least four drawings of each view were made of each nucleus. The area of each drawing was measured with a planimeter. From the areas "en plan" the average area was calculated, and from the areas of the edge views the average thickness was ascertained by dividing each area by its long axis as measured on the drawing, summing the values thus obtained and dividing by the number of drawings measured. The volume of the nucleus was taken as approximately equal to the product of these two calculated quantities: Area and average thickness. The standard deviation of the individual series varied from 2 to 17 percent, giving an average for all series of 8 percent of the measured volume.

In the experiments upon adult interkinetic cells, the cells were selected from culture and placed in a depression slide in culture fluid. They were then stimulated with a glass rod or by rapid suction into, and ejection from, a small pipette until they assumed an approximately spherical shape. When this was done the diameter was ascertained by means of an ocular micrometer and the volume calculated as a sphere. Mononuclear cells with a volume of 0.002 to 0.0035 $mm.^3$ were kept and isolated into small dishes without food and subjected to the chosen reagent.

## EXPERIMENTAL PROCEDURE

All experiments consisted of several

[1] *One drop of culture fluid containing food organisms was added to the 2 cc. of saline in each dish.*

repetitive tests. For the sake of brevity only the aggregate results are presented. The number of tests and the number of amoebae used in the tests are given for each experiment. This combining of results was deemed permissible since in no instance were contradictory results obtained as between tests, although in some tests contradictory behavior obtained as between individual amoebae. Where such behavior was marked, it is noted.

The controls run in all tests were in all instances identical as to the number of amoebae used and the preparation of the cells, and differed only in the absence of the reagent studied. For the sake of brevity the control results are presented in the graphs and not in the text except where no graphed presentation is given as in the results obtained on cells in mitosis.

## THE EFFECT OF LOW OXYGEN TENSION

### Effect on the Fission of Adult Cells

Adult amoebae, selected and isolated as above described, were placed in a glass desiccator, and the air was withdrawn by means of a pump until a pressure of 30 mm. of mercury was registered on an attached manometer. After 24 hours the air was readmitted, the amoebae were removed, and the number of fissions that had occurred ascertained. The amoebae remaining undivided were transferred to dishes containing fresh saline and left for a further 24-hour period under normal atmospheric pressure. At the end of this time the number of fissions was again noted. Five tests were made using 10 amoebae in each test. The results obtained are presented in figure 1, *A*.

It will be seen that during the 24 hours under reduced tension only 2 percent of the amoebae divided. In the second 24 hours at normal tension 46 percent divided, or 48 percent in all at the end of the 48 hours. Attention is called to the rapid recovery of the experimental group after the readmission of air.

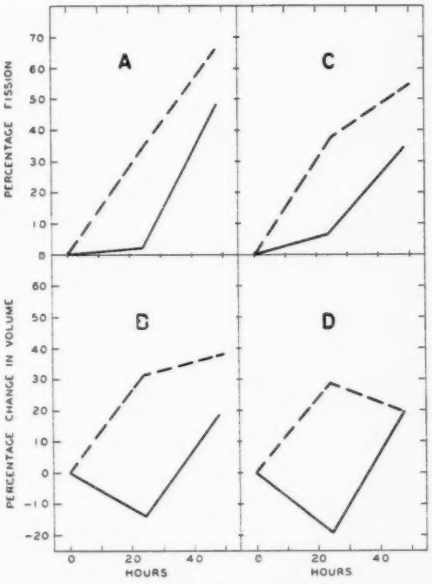



FIGURE 1.—Effect of lowered oxygen tension maintained for 24 hours on: *A*, percentage of fission: *B*, percentage change in nuclear volume. Also effect of lowered oxygen tension together with addition of M/100,000 GSH on: *C*, percentage of fission; *D*, percentage change in nuclear volume in the amoeba. Solid lines, experimental cells; broken lines, controls.

### Effect on Nuclear Growth

Since the nuclei, even in carefully selected young amoebae of known age, vary considerably in volume, the change in volume is given in all instances as percentage change from the original volume of the nucleus.

Amoebae selected at fission and fed for 24 hours as previously described were each, as soon as the nucleus had been measured, placed in a small Pyrex dish containing 0.2 cc. of saline. Each dish was immediately inserted into a Thunberg tube which was rapidly evacuated to a pressure of 30 mm. of mercury. The time at which evac-

uation was made was noted and each tube was opened 25 hours later. Each amoeba was immediately removed and its nucleus was measured. Then it was transferred to another dish containing fresh saline and left for a further 24 hours, when the nucleus was again measured. Five tests, comprising 25 cells in all, were made.

The results are presented in figure 1, *B*. The figure shows that the exposure to the low tension of oxygen resulted in an average decrease in nuclear volume at the end of 24 hours of 14 percent, and that readmission of air resulted at the end of a further 24 hours in a gain of 19 percent above the original volume, or an increase of 33 percent in terms of the original volume above that obtaining at the expiration of the period of exposure to low oxygen tension. As in the experiment on the percentage of fission, the effect of low oxygen tension is reversible upon restoration of the normal tension. This is shown by the resumption of the normal rate of nuclear growth as compared with the rate of growth of the control nuclei during the first 24 hours. This suggests that there is a cessation of growth but no loss of material available for growth during the period under low oxygen tension.

### Effect on the Process of Mitosis

Fifteen amoebae in prophase were selected from culture, washed, isolated into dishes containing saline, and placed in Thunberg tubes. The tubes were evacuated very rapidly by means of a "hyvac" pump, and since the process of mitosis normally takes place in the short time of 35 minutes, the tension was reduced to 10 or 15 mm. of mercury in order to insure very rapid removal of $O_2$ from the cells. Thirty minutes after selection each amoeba was removed from its tube and examined. Of the 15, 1 had divided, 1 was in elongation, and the rest were in late telophase. The controls showed 1 cell divided and the rest in telophase. This demonstrates that the low tension did not interfere with the process of mitosis, per se.

## The Effect of Low Oxygen Tension in Combination with SH Glutathione

To ascertain if the addition of SH glutathione, known to have a stimulative effect under normal oxygen tension, would modify the effect of reduced tension, a series of tests were made identical with those just presented, but with the incorporation in all experimental solutions of SH glutathione in a concentration of M/100,000.

### Effect on the Fission of Adult Cells

Three tests were made using 40 adult cells as experimental material. The results are shown in figure 1, *C*. The curves show that during the 24 hours under low oxygen tension the fission was 7 percent, and during the following 24-hour period under normal oxygen tension the fission reached 35 percent—a result practically identical with that obtained without the added SH glutathione.

### Effect on Nuclear Growth

Five tests were made using 25 amoebae in the experimental solution. Figure 1, *D* shows that the introduction of the SH glutathione produced no alteration of the inhibiting effect of the low oxygen tension on fission. If anything, it tended to increase this effect, the shrinkage of nuclear volume during exposure reaching 19 percent. Restoration of the normal oxygen tension resulted in a gain of volume to 20 percent above the original, or an increase in terms of the original volume of 39 percent above the minimum.

The difference between the decrease in volume in this and in the preceding experiment is not statistically significant, but may indicate that the reducing action of

the SH glutathione served somewhat to enhance the effect of the reduced oxygen tension. The solution at the end of the low $O_2$ period gave a very faint nitroprusside reaction.

### Effect on the Process of Mitosis

Twenty amoebae in prophase were treated as previously described, with the addition of SH glutathione to the medium, and 20 were treated in simple saline as controls. In both lots all were found to be in telophase 30 minutes after selection from the culture, showing that the addition of glutathione did not effect the mitotic process.

## THE EFFECT OF LOWERED PRESSURE COMPARED WITH THE EFFECT OF NITROGEN

To meet the objection that the preceding results were due merely to lowered gas pressure and not to lowered $O_2$ tension four experiments were performed.

In the first experiment 15 adult amoebae, selected as before, were placed in a desiccator, and the evacuation was carried out as described above. After 15 minutes, air was readmitted; 24 hours later the amoebae were removed, and the number of fissions that had occurred were noted. There was no significant difference between the experimental and control cells.

The second experiment was similar to the first, but after evacuation the air removed from the desiccator was replaced by nitrogen. Twenty-four hours later the amoebae were removed from the desiccator and left a further 24 hours in air at normal pressure. There were no divisions during the period of exposure to nitrogen, and only five, or 33 percent, in the 24 hours in air. Thus there was no significant difference from the results obtained with simple evacuation.

The third and fourth experiments were exact repetitions of those previously performed in respect to the effect on nuclear growth, except that the Thunberg tubes after evacuation were filled with nitrogen. The results were within 3 percent of those obtained under simple evacuation.

In view of these results it seemed unnecessary to check the results on mitotic cells, especially when the negative results obtained by Voegtlin and Chalkley (*5*) with CO are considered.

## THE EFFECT OF INCREASED OXYGEN TENSION (24-HOUR EXPOSURE)

### Effect on Fission of Adult Cells

Amoebae were treated exactly as in the previous experiments of this type, but after the desiccator had been evacuated it was refilled with oxygen to a pressure of one atmosphere, reevacuated, and again refilled with oxygen, thus placing the amoebae under practically pure oxygen at a pressure of one atmosphere. The exposure was continued for 24 hours. The

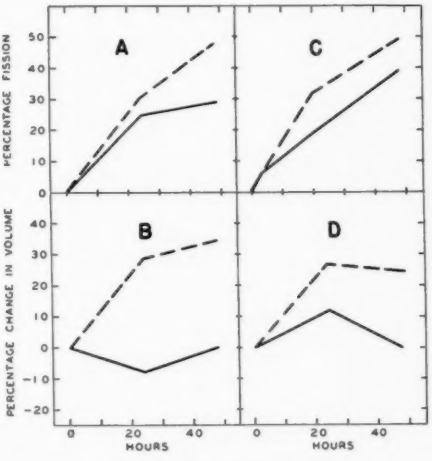



FIGURE 2.—Effect of increased oxygen tension maintained for 24 hours on: *A*, percentage of fission; *B*, percentage change in nuclear volume. Also effect of increased oxygen tension maintained for 3 hours on: *C*, percentage of fission; *D*, percentage change in nuclear volume in the amoeba. Solid lines, experimental cells; broken lines, controls.

subsequent procedure was the same as that in the experiments on the effects of decreased tension. Three tests were made, 10 cells being used in each. The results are presented in figure 2, *A*.

It will be noted that under the high tension of $O_2$ during the first 24 hours fission was a little less than in the controls, 25 percent as compared with 34 percent. This difference is probably not statistically significant. During the second 24 hours after the return to normal oxygen tension, with the fluid environment also rendered normal by a change of saline, there was no recovery of fission rate, such as was obtained on restoration of normal oxygen tension after exposure to a low tension.

#### Effect on Nuclear Growth

The procedure previously used in investigating the effect of low oxygen tension was modified to the extent that the amoebae, after the nuclei were measured, were all put into a desiccator at the same time instead of being put separately into Thunberg tubes. It had become evident that the amount of growth made by the nuclei during the making of measurements was negligible as compared with the changes in volume that occurred during these experiments. Five tests using 5 amoebae in each were run, thus giving 25 experimental cells in all (fig. 2, *B*). It is evident that all growth of the nucleus was inhibited during the exposure and possibly during the succeeding 24 hours at normal tension. The curve shows an 8-percent depression in the nuclear volume during the exposure, but this is approximately the limit of error in measurement and possibly does not represent a real loss. However, as a similar experiment made later showed an almost identical shrinkage of the nucleus, it cannot be completely disregarded. If this shrinkage should be a real one, it follows that there was a slight recovery when the normal oxygen tension was restored.

### The Effect of Increased Oxygen Tension

A series of experiments was performed using the procedure outlined above but limiting the exposure to 3 hours.

#### Effect on Fission of Adult Cells

Four tests were made, using 40 cells in all. Counts of fissions were made at 3, 18, and 48 hours. It will be seen from figure 2, *C* that there is a lessened depression of fission especially during the last 24 hours at normal pressure.

#### Effect on Nuclear Growth

An experiment similar to those previously described, using 25 cells, was run to ascertain the effect on nuclear growth. From figure 2, *D*, it is evident that nuclear growth continued during the first 24 hours and then decreased, but the variation is again practically within the experimental error. Even this short exposure, however, greatly inhibits nuclear growth.

### The Effect of High Oxygen Tension Coupled with Added GSH

The next step was to try the effect of the raised oxygen tension in combination with sulfhydryl. We accordingly repeated the last two experiments but used instead of the plain saline a solution of M/100,000 glutathione made up in the saline.

#### Effect on Fission of Adult Cells

In the first experiment in which exposure to increased oxygen tension was maintained for 24 hours, 3 tests were made using 30 adult cells. It will be seen from figure 3, *A*, that there is no noticeable change from the results obtained with long exposure to increased oxygen tension alone. (See fig. 3, *A*.)

#### Effect on Nuclear Growth

Five tests were made using 25 cells in all, and as shown in figure 3, *B*, the results were almost identical with those obtained

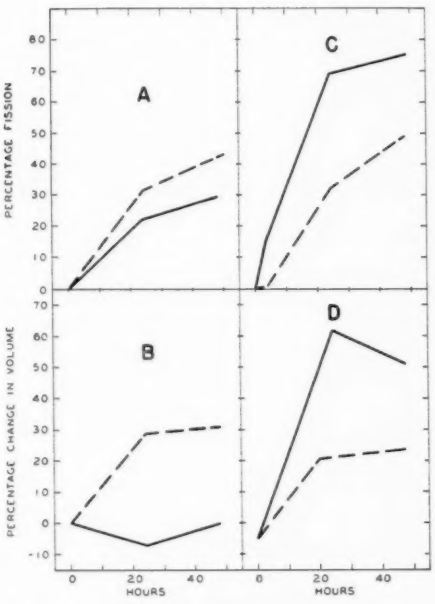



FIGURE 3.—Effect of increased oxygen tension maintained for 24 hours, with addition of M/100,000 GSH on: *A*, percentage of fission; *B*, percentage change in nuclear volume. Also effect of high oxygen tension maintained for 3 hours, with addition of M/100,000 GSH on: *C*, percentage of fission; *D*, percentage change in nuclear volume in the amoeba. Solid lines, experimental cells; broken lines, controls.

in the absence of SH. (See fig. 3, *B*.) It is concluded that the SH does not modify materially the effect of the increased oxygen tension under these conditions.

In the second experiment the exposure was limited to 3 hours and a strikingly different result obtained.

### EFFECT ON FISSION

Six tests were made on interkinetic cells using 60 cells in all. Figure 3, *C*, demonstrates that the addition of glutathione had a marked effect. At the expiration of the first 24 hours, 70 percent of the cells had divided, and after 48 hours, 76 percent. This percentage should be contrasted with the average of 35 percent during the first 24 hours that is regularly obtained for controls.

### EFFECT ON NUCLEAR GROWTH

Five tests using 25 cells were made. The effect of the added glutathione was apparent here also (fig. 3, *D*). During the first 24 hours the average growth of the nuclei was 68 percent. During the next 24 hours, however, the nuclei on an average decreased in volume. Most striking of all, 7 cells (28 percent) underwent fission. The average increase of nuclear volume for these 7 was 89 percent.

It would appear, therefore, that the effect of SH varies from slightly depressing to markedly stimulating, depending upon the length of the exposure to the increased oxygen tension.

## THE EFFECT OF SH AT NORMAL OXYGEN TENSION

The query arose as to what the relation was between this marked stimulation with increased $O_2$ tension for a short period in the presence of added SH, and that obtained on addition of SH without the increased tension. An experiment was therefore performed under the same conditions but at normal $O_2$ tension. It was evident that the status at the end of the first 24 hours was the most valuable for purposes of comparison, and therefore attention was concentrated on this. Six tests, using 60 cells, were made in ascertaining the effect on fission, but only 3 of these tests were carried on for 48 hours. Five tests were made as usual to ascertain the effect on nuclear growth. The results are shown in figure 4, *A* and *B*.

### EFFECT UPON FISSION

The usual stimulation from SH was evident, more than 50 percent of the cells dividing in the first 24 hours.

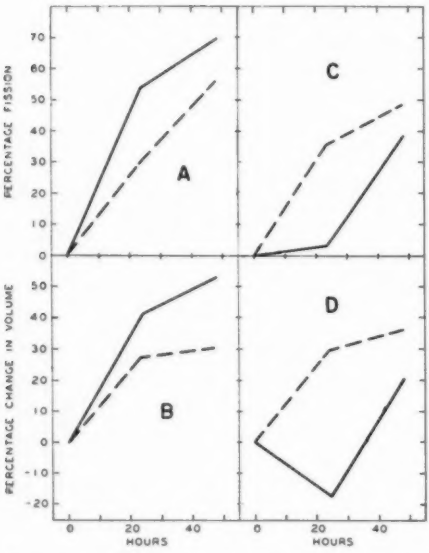



FIGURE 4.—Effect of immersion for 24 hours in M/100,000 GSH solution, on: *A*, percentage of fission; *B*, percentage change in nuclear volume. Also effect of immersion for 24 hours in M/1,000 NaCN solution on: *C*, percentage of fission; *D*, percentage change in nuclear volume in the amoeba. Solid lines, experimental cells; broken lines, controls.

### EFFECT ON NUCLEAR GROWTH

The nuclear growth was stimulated, showing an increase of 42 percent in 24 hours, and reaching 55 percent at the end of 48 hours.

The addition of SH under normal oxygen tension, while stimulating both to fission and nuclear growth, did not have so powerful effect as when supplemented by increased oxygen tension over a short period. The effect attained at the end of 48 hours under normal oxygen pressure, both for fission and nuclear growth, was attained in 24 hours under the increased oxygen tension.

### THE EFFECT OF NaCN

Enzymes of the type of cathepsin and papain are activated *in vitro* not only by SH but also by HCN. Since we were interested in the possible activity of an enzyme of this type being concerned in the growth of the nucleus in the amoeba, we performed an experiment to test the effect of the CN radical. The procedure was the same as that of the last set of experiments on the effect of SH, 4 tests, using 40 experimental cells, were made of the effect on fission, and 4 tests, using 25 experimental cells, were made of the effect on nuclear growth. The results are presented in figure 4, *C* and *D*. It is evident that no stimulative effect obtains. A comparison with the curves for low oxygen tension in plain saline shows a practical identity of results (fig. 1, *A* and *B*), including the reversibility of the depression when at the end of the first 24 hours the cells were transferred to saline without NaCN. It would appear that any possible activating action is completely masked by the well-known inhibition of oxidation by CN. Since the marked inhibition of mitosis by NaCN is well established (*5*), it was not necessary to perform an experiment on that phase.

### THE RELATION BETWEEN THE PERCENTAGE OF FISSION AND THE RATE OF NUCLEAR GROWTH

With respect to the effect of glutathione and copper, Chalkley and Voegtlin (*1*) suggested that the percentage of fission in a given number of cells of similar initial volume during a specified interval was closely correlated with the growth of the nucleus during that period. The experiments just presented provide data suitable for the testing of the general application of this suggestion.

Examination of the figures shows that after the first 24 hours there is a tendency for both the rate of nuclear growth and the percentage of fission to decline. This is, of course, ascribable to the lack of nutriment. It would seem, therefore, that

it may properly be assumed that the most normal cell conditions of the experimental period are those obtaining during the first 24 hours of experimentation. We therefore limited our comparison of data to that period.

If the above-mentioned contention is borne out, a plot of the percentage of fission against the percentage change in nuclear volume should give a relatively smooth curve. The results obtained are shown in figure 5. It is clear that our hypothesis, allowing for some experimental error, appears plausible.

Any hypothesis relating physiological activity such as fission to a simple increase in volume immediately raises the question as to what significance, other than a mere change in water content, it is possible to assign to the volumetric increase. In partial answer to this it may be pointed out that Chalkley (*2*) has shown that the SH content of *Amoeba proteus* is largely concentrated in that part of the nucleus surrounding the chromatin, i. e., the bulkiest portion, that this SH is associated with the protein content of this portion and therefore with material that is probably not diffusible through the nuclear membrane, and finally that there is a cyclic accretion

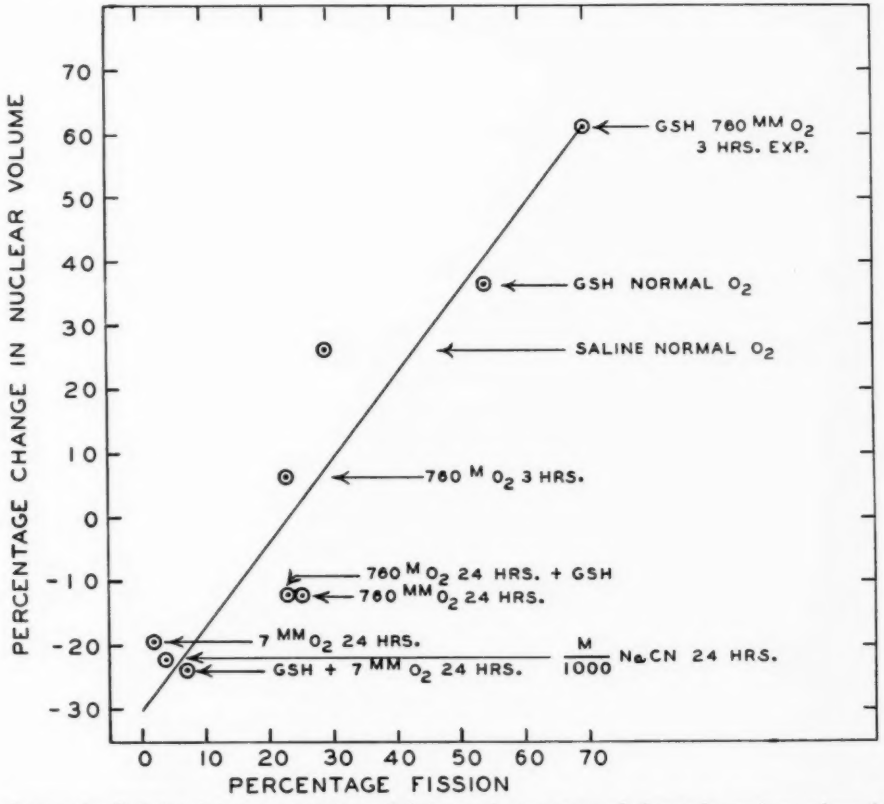



FIGURE 5.—Relation between percentage of fission and percentage of change in nuclear volume in the amoeba, as affected by experimental reagents.

and discharge of this SH carrying material coincident with alternation of interkinesis and mitosis composing the nuclear cycle. This content of SH protein since it increases and decreases coincidentally with the progress of mitosis is possibly of significance with respect to that process. Thus growth of the nucleus, i. e., growth leading to fission is probably a fairly accurate index of the increase in the nuclear content of protein-bound SH as well as the increased water content.

## DISCUSSION

The main experimental results obtained can be summarized as follows: In *Amoeba proteus* both nuclear growth and fission can be retarded either by markedly increasing or by decreasing the $O_2$ tension for a period of 24 hours. The retarding effect of such an increase in $O_2$ tension is unchanged by the addition of M/100,000 SH glutathione to the medium. The retarding effect of this decrease in $O_2$ tension is enhanced by the addition of SH glutathione. While recovery in nuclear growth and fission occurs upon restoration of normal $O_2$ tension after subjection to such lowered tension, this does not obtain in respect to subjection to increased $O_2$ tension. Immersion in M/100,000 SH glutathione for 24 hours at normal oxygen tension results in accelerated nuclear growth and fission, but this acceleration is significantly less than that which obtains if the $O_2$ tension is markedly increased during the first 3 hours of such immersion.

These results indicate strongly that nuclear growth leading to subsequent fission depends on oxidations involving molecular oxygen, since, if the oxygen tension is lowered sufficiently, there is no nuclear growth and consequently no fission, and that this effect is largely reversible on restoration of the normal oxygen tension. That the underlying mechanism is complex, however, is shown by the fact that marked increase in oxygen tension leads to a depression of both nuclear growth and fission unless it is of short duration and is accompanied by an addition of SH. It is not clear from the data whether a large increase in SH would offset the retarding influence to nuclear growth and consequent fission of a prolonged exposure to a very high oxygen tension, but the results suggest that for such growth a balance between the concentration of the SH and oxygen tension is beneficial, if not essential. This may indicate that a certain redox poising capacity in the cell is needed and that in its absence oxidations proceed beyond normal limits with destructive effect. The failure of the nucleus, retarded in growth by prolonged exposure to high oxygen tension, to resume growth upon the restoration of normal tension tends to confirm this assumption. The SH-containing component of the nucleus, if we exclude changes in water content, is the component that furnishes the bulk of the nuclear material. Its increase, together with the increase of the nucleic acid-containing portion which lies within it, probably constitutes the physiologically significant growth of the nucleus. Growth in the organism is the result of synthetic activity regulated by enzymes. It is pertinent, therefore, to inquire how known endocellular enzymes such as nuclease and catheptic proteinase might be expected to be active in furthering the synthesis of protein and nucleic acid-containing components of the nucleus.

If we consider the relation of the nucleus to its environment, it is apparent that there will be available a continuous supply of oxygen from the environment and a continuous supply of protein split products from the food. From Chambers' (*6*) measurements, the pH of both cytoplasm and nucleus will be close to neutrality. It

would thus appear that we have some grounds for the assumption that in the region of the nucleus conditions will obtain that will conform closely to those laid down by Voegtlin, Maver, and Johnson (*3*) for protein synthesis. Voegtlin, Maver, and Johnson state (*3, p. 264*):

> The conditions favoring the synthesis of protein by the proteolytic enzymes of tissues are (*a*) a relatively high oxygen tension; (*b*) a hydrogen-ion concentration not far removed from neutrality; (*c*) a relatively high initial concentration of SH groups attached to proteins or glutathione which can give rise to a relatively high concentration of disulphides; (*d*) a sufficient concentration of suitable protein split products.

With respect to the nucleic acid-containing component Caspersson (*7, p. 139*) asserts that the nucleic acid of the nucleus drops during fission and after fission increases, reaching a maximum just prior to fission. Thus, there is apparently a cyclic variation of the nucleic acid with fission just as Chalkley found for the SH protein component. In respect to the enzymes probably concerned, Maver and Voegtlin (*8*) find that glutathione and cysteine, both SH-containing substances, inhibit the action *in vitro* of tumor nuclease. It is suggestive, therefore, to note that during the growth period of the nucleus in *Amoeba* the arrangement of the chromatin (nucleic acid) it such as to place it within a shell of material having a high SH content.

Let us assume that the growth of the SH-containing component of the nucleus is conditioned by the synthetic activity of a proteinase such as that studied by Voegtlin, Maver, and Johnson (*3*) and that increase in the nucleic acid component is largely governed by a nuclease having properties similar to the nuclease studied by Maver and Voegtlin (*8*) and that the conditions within the cell as to pH, concentration of split products, and SH concentration are as suggested above. On such an assumption and if we bear in mind the spatial distribution of the nuclear components, what might be the result if we subject the *Amoeba* to the experimental procedures reported?

A marked decrease in the oxygen tension would favor proteolysis, especially when the decrease was such as to approximate anaerobiosis (*3*). This might readily result in the enzymic lysis of the proteins of the nucleus, but it should be noted that it probably would not immediately result in lysis of the nucleic acid component (the chromatin) since a low oxygen tension would be favorable to its maintenance. Maver and Voegtlin (*8*) found that anaerobic conditions inhibit the lytic activity of nuclease. Hence, since the fundamental genetic structure would be undamaged, it is probable that even after relatively prolonged exposure to such conditions recovery of nuclear growth would occur upon restoration of normal oxygen tension. To add SH to the system under conditions of low oxygen tension would tend merely to increase the effectiveness of the anaerobiosis through competitive withdrawal of oxygen from the surrounding medium. If the tension of oxygen is markedly increased and maintained for a short period, and particularly if SH is added, an increase in protein synthesis may be expected, since such would constitute a mild intensification of the conditions suggested by Voegtlin and Maver to favor synthesis. It must not be overlooked, however, that such conditions favor lysis of the nucleic acid component unless sufficient SH is present to inhibit.

The effect of overoxygenation, it is to be noted, appears as the one instance in the experimental data where fission continued for some time, though at a slower rate, while nuclear growth was strongly retarded or reversed. Voegtlin, Maver, and Johnson (*3*) found in some cases that very

strong oxygenation of their *in vitro* systems containing tissue extract, after inducing an initial synthesis produced, if long continued, a strong lysis. This effect was emphasized if copper was added. It has been pointed out by Dr. Maver (personal communication) that there is evidence that overoxidation of papain, an enzyme of the catheptic type, results in a blocking of its activity probably because of oxidation of the SH groups contained therein. It is reasonable to assume that traces of heavy metals such as copper are present in *Amoebea*, and we are proceeding on the assumption that catheptic enzymes are involved in nuclear growth. It seems, therefore, that on strong and long-continued oxygenation of the cell the effect might well be an initial stimulation of metabolic activity resulting in increased synthesis, rapidly followed by protein break-down, and finally by the destruction of the enzyme system. Such disappearance of the SH component of the nucleus would in all probability expose the nucleic acid component in turn to oxygenation which, from the findings of Maver and Voegtlin (*8*), Edlbacher and Kutscher (*9*), Waldschmidt-Leitz, Scharikowa, and Schaffner (*10*), might be expected to lead to its lysis. As a result there would occur a condition in which the SH-containing material (protein) had been broken down, in which the enzyme system necessary for its resynthesis had been damaged, and a consequent splitting of the nucleic acid component (chromatin) had occurred. A situation would thus be produced which would render recovery of the status quo by the cell extremely difficult. This step-by-step process would be expected to be slow, and, as pointed out, the destruction would be preceded by a spurt of stimulation. The rate of fission might therefore appear experimentally to be less retarded than the nuclear growth.

This, as previously pointed out, would not obtain in the case of oxygen withdrawal since this would tend to preserve the nucleic acid component and merely shift the balance of the catheptic enzyme system toward lysis. These changes would leave the system easily reversed in activity by restoration of the normal condition of oxygen tension, and there would appear no initial spurt of stimulation which could produce experimentally sustension of fission rate.

This discussion is admittedly highly speculative, but it seems suggestive that not only do the types of substance known to occur in the nucleus fit into the picture presented, but also that their demonstrated spatial arrangement conforms to the scheme.

The hypothesis presented suggests that further experimentation with respect to variation in nuclear growth and fission under different conditions of oxygen tension, of the SH content of the nucleus, and with respect to the distribution of nucleic acid and conformation of the chromatin arrangement under such conditions will prove of value. Of particular interest, too, should be studies of the effect of known components of the oxidative enzyme systems of cells such as Keilin's cytochrome, Warburg's flavin enzyme, and cozymase.

The data as a whole strongly suggest that the physiological role of the SH group is to act in conjunction with oxygen in the regulation of the activity of enzymes, particularly those concerned with nuclear growth.

## REFERENCES


(*1*) CHALKLEY, H. W., and VOEGTLIN, CARL: The chemistry of cell division. III. Inhibition of cell division of *Amoeba proteus* by high dilutions of copper salts—Antagonism of copper and glutathione. Pub. Health Rep., **47**: 535–560 (1932).

(*2*) CHALKLEY, H. W.: The chemistry of cell division. VII. The distribution of sulphydryl in *Amoeba proteus* in interkinesis and mitosis, as shown by the nitroprusside test. Protoplasma, **28**: 489–497 (1937).

(*3*) VOEGTLIN, CARL, MAVER, MARY E., and JOHNSON, J. M.: The influence of the oxygen tension on the reversal of proteolysis (protein synthesis) in certain malignant tumors and normal tissues. J. Pharmacol. & Exper. Ther., **48**: 241–265 (1933).

(*4*) CHALKLEY, H. W., and DANIEL, G. E.: The relation between the form of the living cell and the nuclear phases of division in *Amoeba proteus* (Leidy). Physiol. Zool., **4**: 592–619 (1933).

(*5*) VOEGTLIN, CARL, and CHALKLEY, H. W.: The chemistry of cell division. IV. The influence of $H_2S$, HCN, $CO_2$ and some other chemicals on mitosis in *Amoeba proteus*. Protoplasma, **24**: 365–383 (1935).

(*6*) CHAMBERS, R.: The nature of the living cell as revealed by micromanipulation. *In* Alexander's Colloid Chemistry, vol. 2. The Chemical Catalog Co., New York (1928). See p. 480.

(*7*) CASPERSSON, TORBJÖRN: Über den chemischen Aufbau der Strukturen des Zellkernes. Skandinav. Arch. f. Physiol., **73**, Suppl. **8**, 154 pp.

(*8*) MAVER, MARY E., and VOEGTLIN, CARL: The chemistry of cell division. V. The influence of ascorbic acid, glutathione and cysteine on the activity of tumor nuclease. Am. J. Cancer, **25**: 780–788 (1935).

(*9*) EDLBACHER, E., and KUTSCHER, W.: Über den Stoffwechsel der Tumoren. III. Mitteilung. Beiträge zur Kenntnis der Phosphatasen. Ztschr. f. physiol. Chem., **207**: 1–15 (1932).

(*10*) WALDSCHMIDT-LEITZ, E., SCHARIKOVA, A., and SCHAFFNER, A.: Über den Einfluss von Sulfhydrylverbindungen auf enzymatische Prozesse. Ztschr. f. physiol. Chem., **214**: 75–88 (1933).

# CHEMICAL STUDIES ON THE COMPONENTS OF NORMAL AND NEOPLASTIC TISSUES. I. VISCOSITY AND STREAMING BIREFRINGENCE OF SODIUM THYMONUCLEATE

By JESSE P. GREENSTEIN, *research fellow*, with the technical assistance of WENDELL V. JENRETTE,
*National Cancer Institute, National Institute of Health, United States Public Health Service*

## INTRODUCTION

An important approach to the study of living tissues consists of the investigation of the separate chemical components of the tissues by such means as to characterize them adequately in terms of structure, physicochemical behavior, and possible physiological function. Among the components of animal and plant cells the nucleic acids occupy a position of unusual interest, in part because of their special position within the nucleus with its consequent relation to specific nuclear processes (*1*), and in part because of certain similarities in structure with numerous oxidative co-catalysts (*2*). In the course of transformation of normal tissues to malignant, the possibility exists that alterations in the chemical components of the tissues may precede or accompany such transformations. Since presumably growth processes are controlled by the nucleus (*3*), it would appear that a comparative study of the nuclear components of normal and tumor tissues is desirable.

Before the components of cancerous tissues may be adequately characterized, however, it is necessary to possess information concerning the analogous components of normal tissues. In the case of nucleic acids, the intramolecular structure has been investigated by a host of workers, notably Levene (*4*), Thannhauser and Angermann (*5*), Feulgen (*6*), Bredereck (*7*), and Caspersson (*8*), whereas studies of their physical properties are very meager. Hammarsten (*9*) described certain of the physicochemical properties of thymonucleic acid, and later in a brief note with Signer and Caspersson (*10*) made the observation that sodium thymonucleate was highly polymerized and showed intense double refraction of flow in aqueous solution.

In the endeavor to characterize this important susbtance more fully, the present investigation on certain of the specific properties of sodium thymonucleate from normal calf thymus was undertaken. In particular, the effect of various salts on the viscosity and the streaming birefringence of this substance was studied, and a definite correlation between these properties was found. In addition, the effect of various salts on the properties of nucleic acid-protein mixtures was investigated in order to make possible subsequent comparisons with nucleoproteins derived from various tissues.

## SIGNIFICANCE OF DOUBLE REFRACTION OF FLOW

It has long been known that certain types of tissues, such as nerve fibers, smooth muscle, and the dark bands of striated muscle, are optically anisotropic, i. e., the velocity with which a beam of light passes through the tissue depends on the direction of incidence of the beam. Since the index of refraction is inversely proportional to the velocity with which light travels through the substance, it is possible to obtain different indices of refraction for the emergent beams with

different directions of the incident light. This phenomenon is known as double refraction.

It has been shown that double refraction in tissues is due to the presence of a definite pattern of oriented elements in the tissue (*11*). These elements are generally long, rod-shaped, or asymmetric molecules oriented in parallel. When the tissue is extracted by a suitable solvent, these elements pass into solution and the ordinary random thermal agitation within the solution prevents the orientation characteristic of the intact tissue. When, however, the solution is stirred, the dissolved elements are oriented by the shearing forces arising during flow and the solution becomes doubly refracting. This effect is known as double refraction of flow, or streaming birefringence. The extent of the streaming birefringence depends upon the shearing stress exerted and reaches an apparent upper limit when the dissolved particles are completely oriented. The shearing force necessary for the orientation is in turn dependent upon the shape of the particles in solution—the more asymmetric the shape, the less force is required. Thus, a solution of egg globulin or fibrinogen requires high stirring speeds in order to demonstrate double refraction of flow; on the other hand, a solution of myosin or sodium thymonucleate needs only to be stirred gently by hand with a glass rod to demonstrate the effect.

This behavior of myosin has been described by Edsall and Mehl (*12*). We have found the same to be true of sodium thymonucleate and have made an extensive study of certain aspects of this property. The methods employed in this study at the present time on the thymonucleate from normal tissues, will be repeated with material isolated subsequently from various tumor tissues, and the results will be compared.

## EXPERIMENTAL PROCEDURE

### THE PREPARATION OF SODIUM THYMONUCLEATE

The Feulgen-Levene procedure for the isolation of nucleic acid from tissues consisted in heating the latter with strong alkali followed by acidification, filtration of the denatured proteins, and precipitation of the nucleic acid in the filtrate with alcohol. The several preparations from calf thymus which we obtained by this method were invariably contaminated with protein and were slightly colored.[1] Moreover, they were soluble in water only on heating, and on cooling the solution frequently gelled. This type of preparation, while apparently quite suitable for studies of intramolecular structure (*4*), was clearly inappropriate for physicochemical investigations. Consequently the newer preparation of calf thymus nucleate by the Hammarsten-Bang procedure was employed (*9*). This procedure leads to a pure white, practically protein free preparation, which separates from solution on treatment with alcohol as long fibers of a peculiar asbestoslike appearance. This material was easily soluble in cold water, yielding clear viscous solutions which showed intense double refraction of flow. Two preparations of sodium thymonucleate according to Hammarsten were made (preparation No. 1, N 15.0, and P 8.5; preparation No. 2, N 15.1, P 8.8). Although both preparations were made in as nearly identical a fashion as was possible, the values for the viscosity and double refraction of flow were appreciably different for the two preparations (table 1). It is possible that very slight variations in the preparative procedures may be reflected in considerable differences in the physical constants of different products, even though the

[1] *Hyflow supercel was used to clarify the hot tissue extracts instead of the colloidal iron recommended by Levene* (*4*).

analytical data are the same for all of them. The difficulty in obtaining different preparations of substances of very high molecular weight which yield exactly reproducible physical values is recognizably great (*12*, *13*).

TABLE 1.—*The viscosity and streaming birefringence of aqueous solutions of sodium thymonucleate as a function of concentration*

[Temperature 30° C.]

| Preparation No. | Concentration | Specific viscosity[1] | $\nu_\infty$ | Streaming birefringence |
|---|---|---|---|---|
| | *Percent* | | | |
| 1 | 1.0 | 12.55 | 2024 | +++ |
| | .5 | 6.53 | 2106 | ++ |
| | .25 | 3.54 | 2284 | + |
| | .10 | 1.52 | 2451 | − |
| 2 | .5 | 7.46 | 2407 | ++++ |
| | .25 | 4.43 | 2858 | +++ |
| | .10 | 2.08 | 3355 | ++ |

[1] Extrapolated from the viscosity $\bar{\beta}$ curves for very high values of $\bar{\beta}$.

A comparison between solutions of the Feulgen-Levene and the Hammarsten-Bang preparations of calf thymonucleate revealed that the birefringence and viscosity of the latter preparations were approximately three to five times greater than the former. These results are consistent with the experiments of Hammarsten (*9*) in which he described the deleterious effects of alkali on the physical properties of sodium thymonucleate (cf. Schmidt and Levene (*14*)). On the other hand, the relative effect of various salts on the viscosity and streaming birefringence of both types of preparation, described later, were essentially similar.

It may be emphasized that we are dealing here with the completely neutralized tetrasodium salt of nucleic acid (*9*). The aqueous solutions of this salt are very slightly on the acid side of neutrality. Solutions of the free acids are too unstable to be kept for long.

## THE MEASUREMENTS OF DOUBLE REFRACTION OF FLOW AND OF VISCOSITY

### DOUBLE REFRACTION OF FLOW

The streaming birefringence of solutions of sodium thymonucleate was observed between crossed Polaroid disks in the apparatus described by Edsall and Mehl (*12*). When the solutions were at rest the field was uniformly dark; stirring of solutions of moderate concentration caused the field to brighten; and when a rotary motion was applied, a dark cross of isocline appeared against the lighted field. When the stirring suddenly ceased, the cross of isocline very quickly vanished and the field almost immediately darkened. This is in marked contrast with the behavior of a comparable solution of rabbit myosin, because in the latter the cross of isocline persists for an appreciable length of time after stirring has ceased ((*12*) and personal observations). In general, the addition of salts to a solution of sodium thymonucleate resulted in a diminution in the intensity of the streaming birefringence. With high concentrations of certain salts this property completely disappeared. The concentrations of salt just necessary to destroy the double refraction depend upon the nature of the salt and upon the concentration and type of preparation of the sodium thymonucleate.[2] Obviously the greater the concentration of the latter and the greater its intensity of birefringence the higher will be the concentration of salt necessary to destroy the birefringence. The first investigation of these phenomena was conducted on a 0.5-percent solution of sodium thymonucleate prepared according to Levene (*4*). The following results describe the concentration in millimols

[2] *We mean by the phrase, "to destroy the double refraction," only that the double refraction has been reduced to such an extent as to be no longer observable by means of the apparatus used.*

added per gram of solution necessary to destroy instantly the double refraction of the nucleate solution; guanidine HI 1.1, guanidine HSCN 1.2, guanidine HBr 1.4, KI 2.4, NaSCN 3.0, guanidine HCl 3.2, KBr 4.2, urea 12.4. The influence of the nature of the salt is clearly evident. The remarkable effect of the guanidinium ion is especially noteworthy and will be discussed in more detail later. It may be pointed out that the concentrations of the salts mentioned above are much lower than those necessary to destroy the double refraction in sodium nucleate solutions prepared according to Hammarsten. (See table 2.)

TABLE 2.—*The viscosity and streaming birefringence of solutions of sodium thymonucleate in various salts*

[The nucleic acid solution was 0.5 percent. Temperature 30° C.]

| Preparation No. | Salt | Concentration of salt | Specific viscosity[1] | $\eta_\infty$ | Streaming birefringence |
|---|---|---|---|---|---|
| | | *mM added per cc. of solution* | | | |
| 1 | | | 6.53 | 2106 | ++ |
| | NaCl | 1.0 | 4.52 | 1458 | + |
| | NaBr | 1.0 | 4.34 | 1400 | + |
| | NaI | 1.0 | 4.17 | 1345 | + |
| | Guanidine HCl | 1.0 | 4.19 | 1351 | + |
| | Urea | 1.0 | 5.23 | 1687 | + |
| | Glycine | 1.0 | 4.92 | 1587 | + |
| | NaCl | 4.0 | 3.14 | 1013 | + |
| | NaBr | 4.0 | 2.86 | 923 | + |
| | NaI | 4.0 | 2.48 | 800 | + |
| | Guanidine HCl | 4.0 | 2.63 | 848 | + |
| | Urea | 4.0 | 3.81 | 1229 | + |
| | NaBr | 6.0 | 2.30 | 742 | + |
| | NaI | 6.0 | 1.71 | 551 | − |
| | Guanidine HCl | 6.0 | 2.27 | 732 | + |
| | Urea | 6.0 | 3.66 | 1180 | + |
| | NaBr | 8.0 | 2.01 | 648 | + |
| | NaI | 8.0 | .90 | 290 | − |
| | Guanidine HCl | 8.0 | 1.72 | 555 | − |
| | Urea | 8.0 | 3.34 | 1077 | + |
| 2 | | | 7.49 | 2416 | ++++ |
| | Guanidine HCl | 4.0 | 4.53 | 1461 | ++ |
| | Urea | 4.0 | 6.67 | 2151 | +++ |
| | Guanidine HCl | 8.0 | 3.12 | 1007 | + |
| | Urea | 8.0 | 5.64 | 1819 | ++ |

[1] Extrapolated from the viscosity-$\bar{\beta}$ curves for very high values of $\bar{\beta}$.

Particularly striking is the ready reversibility of the salt effect on the streaming birefringence. An apparently completely isotropic solution of sodium thymonucleate was prepared by adding any one of the above-mentioned effective salts; when such a solution was either dialyzed until salt-free, or else treated with excess alcohol to precipitate the nucleate and the latter further purified by frequent precipitation, the resultant aqueous solution in either case showed nearly the same intensity of double refraction as did the original solution of nucleate without salt. This reversibility has been repeatedly demonstrated. It stands in marked contrast with the behavior of myosin, for apparently it has not been possible as yet to restore the double refraction of solutions of this protein when once lost (*12*).

## VISCOSITY

The relative viscosities of solutions of sodium thymonucleate were measured at 30° C. ±0.01 in a capillary viscometer of the Bingham and Jackson type (*15*). All measurements were made under known external pressures which permitted various rates of flow of the solutions in the viscometer. The pressures were varied by means of compressed air and a system of water traps and were measured by either a water or a mercury manometer depending upon the magnitude of the pressure employed (*16*). The radius of the capillary, 0.047 cm., was found by measuring the time of flow of water under a known pressure and subsequently applying Poiseuille's law. In this connection, the value of Bingham and Jackson (*15*) for the absolute viscosity of water at 30° was adopted. The volume of fluid used in the viscometer was always 5.0 cc. The salts studied were NaCl, NaBr, NaI, guanidine HCl, urea, and glycine. Two different preparations of sodium thymonucleate, made according to Hammarsten, were used, preparations Nos. 1 and 2.

The salt solutions were prepared by adding the dry salt in various quantities

to a known volume of an aqueous solution of sodium thymonucleate. Similar solutions of the salts in distilled water were made for comparison. The densities of the salt solutions were not determined since relative measurements were made, and the contribution of the sodium nucleate to the density was quite negligible in comparison with that of the salt. *Fresh solutions were invariably prepared before every run.*

The pressure-time products for the salts in water were quite constant over the range of pressures employed (*17*). The values of this product for the same concentration of salts were different, however, for different salts. At comparable concentrations the order of magnitude was as follows: glycine> NaCl> NaBr>NaI> guanidine HCl> urea. The data for the sodium salts are consistent with those of Getman for the corresponding ammonium halides (*18*).

The pressure-time product for solutions of sodium thymonucleate in water, however, were not independent of the applied pressure except for either very dilute solutions or for very high pressures. In short, solutions of thymonucleate in water do not follow Poiseuille's law. They exhibit structural viscosity to a marked degree. The progressive addition of salts to such solutions results in a distinct and progressive decrease in the viscosity.[3] Certain salts in sufficient concentration are capable of destroying entirely the structural character of the viscosity. When this phenomenon occurred and the resulting solution of sodium nucleate and salt apparently followed Poiseuille's law, it was found that the streaming birefringence, like the structural viscosity, had completely disappeared. The two properties of streaming birefringence and structural viscosity thus appear to be closely interrelated, and may be thought of as being due to a high degree of molecular asymmetry of the sodium thymonucleate. Signer, Caspersson, and Hammarsten (*10*) give an axial ratio (the ratio of the long and short axes of an ellipsoid of revolution) for this substance of 300:1, but in the absence of experimental data it is not possible to see how they arrived at this conclusion. Myosin is also a highly asymmetric molecule (*11*), and as Edsall and Mehl (*12*) show, there is a close relationship between the streaming birefringence of this substance and its structural viscosity.

Several investigators (*19*, *20*, *21*, *22*, *23*, *24*, *25*, *26*) have indicated that there is some connection between the shape of molecules and the viscosity of their solutions, although no completely satisfactory relationship has as yet been developed. In order to formulate the present data to serve for future theoretical discussion, the experimental findings are given in terms of a quantity $\nu$ which is the ratio of the specific viscosity, $\eta sp$, to the volume fraction $\phi$ of the thymonucleate.

$$\nu = \frac{\eta sp}{\phi} = \frac{1}{\phi}\left(\frac{\eta}{\eta_o} - 1\right)$$

($\eta$ is the viscosity of the nucleate or nucleate-salt solution, $\eta_o$ that of water or salt water as the case may be: $\frac{\eta}{\eta_o}$ is thus the relative viscosity).

Einstein calculated that for a solute made up of incompressible, uncharged spheres $\nu$ should be 2.5. Hatschek (*27*) and recently Polson (*28*) showed that the experimentally found values of $\nu$ were nearer 4. However, for nonspherical molecules this quantity is greater than 2.5, owing to the additional work necessary to rotate the molecule in the hydrodynamic field; and the more the shape of the molecule departs

[3] *Hammarsten* (*9*) *showed clearly that salts will lower the viscosity of sodium thymonucleate solutions. His data were apparently obtained at a single pressure and were not sufficiently extensive to reveal the effects brought about by different salts.*

from that of a sphere, i. e., the greater the asymmetry, the greater must be the additional work. The measured apparent viscosity for substances of this type will consequently vary with the external pressure applied to force them through the capillary of the viscometer. At low external pressures the measured viscosity will be appreciably greater than that measured at high pressures because of the random orientation of the molecules under the former condition. At high pressures, however, the molecules will be oriented by the shearing stresses in the liquid and $\nu$ will tend asymptotically to a lower limiting value $\nu_\infty$.

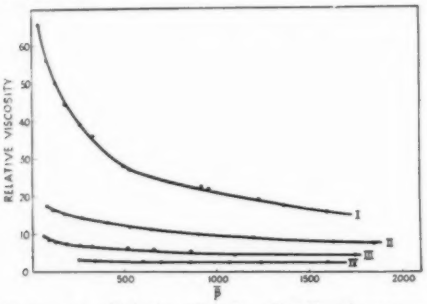



FIGURE 1.—Relative viscosity of sodim thymonucleate in water (preparation No. 1) as a function of the velocity gradient. I, 1.0 percent; II, 0.5 percent; III, 0.25 percent; IV, 0.10 percent.

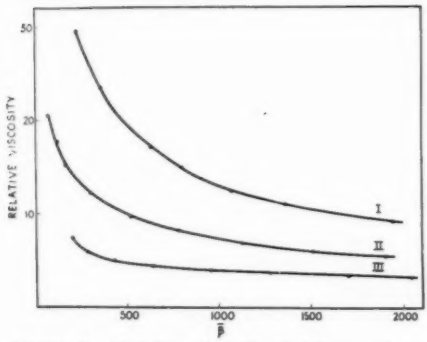



FIGURE 2.—Relative viscosity of 0.5-percent sodium thymonucleate in water (preparation No. 2) as a function of the velocity gradient. I, 0.5 percent; II, 0.25 percent; III, 0.10 percent.

In order to formulate the experimentally found values in terms of $\nu_\infty$, it is necessary to have further information in respect to two quantities: (1) The partial specific volume of sodium thymonucleate; and (2) the velocity gradient in the capillary. For the latter we have followed Edsall and Mehl (*12*) in employing the function of Kroepelin (*29*). Kroepelin describes a mean velocity gradient, $\bar{\beta}$, as a function of the volume $\nu$ of liquid flowing through the capillary in time $t$ and of the capillary radius $r$.[4]

$$\bar{\beta}=\frac{8\nu}{3\pi r^3 t}$$

For the determination of the partial specific volume of sodium thymonucleate we measured the densities of solutions of this substance at 30° in pycnometers, and obtained the specific volumes from the relation:

$$D=d+\frac{(1-\alpha d)C}{100}$$

where $D$ is the density of the solution and $d$ of the solvent, $\alpha$ is the partial specific volume, and $C$ is the concentration in grams per 100 gm. of solution. For solutions of the following concentrations the respective values of $\alpha$ were found: 2.0 percent, 0.65; 1.0 percent, 0.57; 0.5 percent, 0.65; 0.25 percent, 0.64: and 0.125 percent, 0.59. The average value of $\alpha$ is 0.62, and this was used in all computations of $\phi$ from the experimental data.

### THE PROPERTIES OF SODIUM THYMONUCLEATE IN WATER

The structural viscosity of solutions of sodium thymonucleate in water for preparations Nos. 1 and 2 is clearly shown in figures 1 and 2, respectively. The viscosity

[4] *Edsall and Mehl (12) point out that the magnitude of $\nu$ is a function of the ratio of the velocity gradient, $\beta$, to $\Theta$, the rotary diffusion constant (30, 31). The latter quantity has not yet been determined for sodium thymonucleate, but the results in the present paper suggest that it must be relatively small.*

falls off with increasing velocity gradient and appears to approach a limiting lower value. This limiting value minus 1 divided by the volume fraction of thymonucleate in solution was taken as equivalent to $\nu_\infty$ as defined above. Thus:

$$\nu_\infty = \left(\frac{\eta sp}{\phi}\right)_{\beta \to \infty}$$

Values of the specific viscosity and $\nu_\infty$ for both preparations are given in table 1. In the last column of the table are given the relative intensities of the streaming birefringence of each solution as visually estimated. The values of $\nu_\infty$ and the apparent intensity of the double refraction are different for the two preparations, but it is quite clear that the more viscous preparation (No. 2) possesses also a more intense birefringence. Both preparations consistently show the singular phenomenon of an apparent increase in $\nu_\infty$ with decreasing concentration of nucleate. In the complete absence of intermolecular forces, the value of $\nu_\infty$ should be independent of the solute concentration. It is difficult, however, in the present instance to interpret the change of $\nu_\infty$ with concentration, and in the absence of other information further discussion of this point must be postponed. The very large values of $\nu_\infty$ for both preparations suggest a high degree of asymmetry of the solute molecules. Comparison with myosin (*12*) reveals that $\nu_\infty$ for the thymonucleate is approximately 10 times as great as for the protein. It is difficult to conceive that this difference is entirely owing to differences in the magnitude of the axial ratios of the molecules of the two substances. Other factors, notably solvation, an electroviscous effect, and perhaps intense intermolecular forces may contribute to the considerable difference between the two substances. In any event, the fact that myosin is a protein and sodium thymonucleate a complex salt imposes caution on attempts to draw too close analogies, even though some striking parallelisms are present.

### THE PROPERTIES OF SODIUM THYMONUCLEATE IN SALT SOLUTIONS

The profound effect which salts exert on reducing the viscosity of sodium thymonucleate is illustrated in figures 3, 4, and 5.

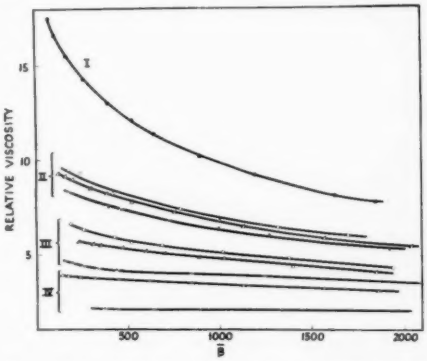



FIGURE 3.—Relative viscosity of 0.5-percent sodium thymonucleate (preparation No. 1) in salt solutions as a function of the velocity gradient. I, in water alone; II, in 1M salt; III, in 4M salt; IV, in 8M salt.

○NaCl, △NaBr, × NaI

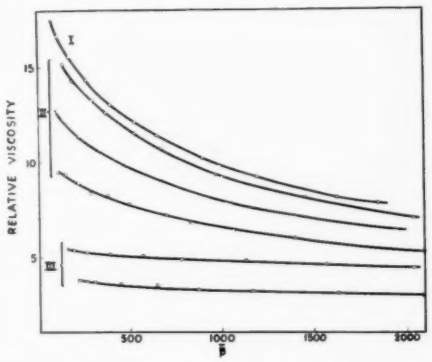



FIGURE 4.—Relative viscosity of 0.5-percent sodium thymonucleate (preparation No. 1) in urea, glycine, and guanidine HCl as a function of the velocity gradient. I, in water alone; II, in 1M salt; III, in 8M salt.

◇ Urea, □Guanidine HCl, +Glycine

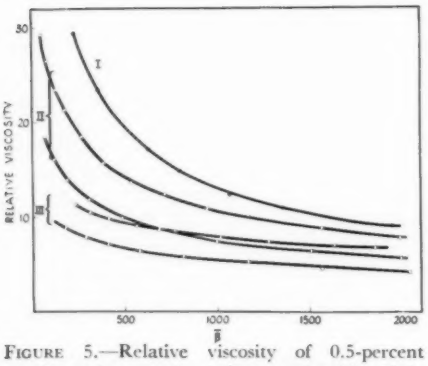



FIGURE 5.—Relative viscosity of 0.5-percent sodium thymonucleate (preparation No. 2) in urea and guanidine HCl as a function of the velocity gradient. I, in water alone; II, in 4M salt; III, in 8M salt.

◇ Urea, □ Guanidine HCl

The pertinent data are shown in table 2. The curves for the effect of 6M salts on preparation No. 1 have been omitted from the graph (fig. 3) to avoid confusion because of overlapping. There is a progressive decrease in the value of $\nu_\infty$ for thymonucleate accompanied by an apparent decrease in the intensity of birefringence as the concentration of salt increased. In the presence of 6M NaI or 8M guanidine HCl, both the structural character of the viscosity and the double refraction disappeared (table 2). The value of $\nu_\infty$ under these conditions decreased to about one-fourth of its value in water; in 8M NaI it dropped to about one-seventh. The data for both preparations Nos. 1 and 2 showed the general properties of the salts, but the extent of the effect was smaller for preparation No. 2 which possessed an originally greater viscosity and a more intense birefringence. At each concentration, from 1M to 8M, the relative effect of the various salts in diminishing the specific properties of the thymonucleate solution remained the same. At comparable concentrations, the order of effectiveness in this respect was as follows: NaI>guanidine HCl> NaBr> NaCl> glycine>urea. These results are consistent with those obtained with sodium thymonucleate prepared according to Levene and described above (p. 80).

Examination of the curves in figure 3 and the data in table 2 reveals that the quantitative differences between the various salts invariably tend to increase as the concentration of the salts increase. Whereas, for example, at 1M there is almost no difference between the effect of NaI and guanidine HCl, at 4M the difference is appreciable, and at 8M the effect of NaI is nearly double that of guanidine HCl. At very low concentrations of inorganic salts, as Hammarsten has indicated (*9*), it is likely that individual differences between salts of the same valence type will disappear. The influence of specific ions is, however, most clearly revealed at high concentrations of salts. It must be borne in mind that the concentration of thymonucleate employed in these studies is also comparatively high.

A comparison of the effect of various salts on diminishing simultaneously the viscosity and double refraction of myosin (*12*) with the data in this paper on sodium thymonucleate reveals some striking parallelisms. When the cation is the same, the iodide is stronger than the bromide, and the latter is more effective than the chloride. The most effective monovalent cation in diminishing the specific properties of both myosin and thymonucleate appears so far to be guanidinium. Guanidine HCl is consistently more effective than urea. Glycine affects both thymonucleate and lobster myosin.[5] [6] However, it must

[5] *The remarkable effect of the guanidinium ion not only on thymonucleate but also on many proteins is especially noteworthy (32–35). The guanidine group is present in a wide variety of biological materials, ranging from small molecules like creatine to large molecules like proteins. In all of them, because of its alkaline dissociation constant, the group exists as the guanidinium ion. The association of the nucleic acid with substances like the protamines, which are rich in guanidine groups,*

be pointed out that far higher concentrations of salts are necessary to produce the same effects in thymonucleate than in myosin. The lability of the latter to denaturing agents is very much greater than that of either the former or of the tobacco mosaic virus protein (*36*).

Edsall and Mehl (*12*) have interpreted the effect of the various salts on myosin as being due to a dissociation of the protein molecule into smaller, less asymmetric particles. The same explanation, in part, may be tentatively applied to the analogous case of sodium thymonucleate. The latter substance has a very high molecular weight, estimated to be in the neighborhood of $1\times10^6$ (*1*). The molecular weight of the average tetranucleotide calculated from its components amounts to about 1,400. It would therefore appear that the molecule of thymonucleate is highly associated. If the forces of association were largely of a saltlike character, i. e., between phosphate and purine amino groups, it would be expected that the addition of ionizable salts would be capable of dissolving such linkages. The comparatively strong effect of glycine and urea, however, makes such an explanation not wholly applicable. The association forces in sodium thymonucleate in any event are apparently very strong, for the latter substance is able to preserve its specific properties to some degree under such drastic conditions as boiling in alkali. Moreover, when these properties disappear in the presence of certain salts, they may be fully restored by simply removing the salt. Myosin does not possess this reversibility, and to preserve its specific properties great care must be exercised in handling the protein. It would be of great interest to investigate the possible changes of thymonucleate in particle size and shape by means of the ultracentrifuge, and plans for a study of this sort are already under way.

### NUCLEIC ACID-PROTEIN MIXTURES

For the greater part, if not entirely, nucleic acid exists in the tissues in some form of combination with protein. Whether this combination is of a saltlike character, or whether it is due to an ester linkage is as yet unknown and may well be different for different proteins. The method of separation of nucleic acid from the protein offers some clue to the type of combination. For example, in the Hammarsten-Bang procedure, the neutral thymus nucleoprotein solution is saturated with NaCl and the protein is precipitated as the chloride while the sodium nucleate remains in solution. This is obviously a metathetical reaction, and it is difficult to conceive of more than a possible saltlike combination between nucleic acid and protein in thymus nucleoprotein. Further discussion of the tissue nucleoproteins will be reserved for subsequent papers.

In the investigations reported in the present paper, the behavior of mixtures of protein with thymonucleic acid and with various salts was studied. The protein employed was rat serum albumin, purified through several crystallizations with ammonium sulfate. The final solution of the protein in water was dialyzed until free of ammonium sulfate and was adjusted to 2.8-percent concentration. Aliquots of this solution were treated with definite amounts of sodium thymonucleate or nucleate plus salts, as the case might be.

*invites speculation. At the present time, however, it is thought best to call attention to the properties of this ion and to postpone further discussion until more experimental studies are available.*

[6] *Stanley and Lauffer (36) have shown that the double refraction of the tobacco mosaic virus protein in 6M urea falls to about one-third the original value in 2 hours. Frampton (13) has demonstrated the effect of urea in diminishing the viscosity of this protein. The behavior of the tobacco mosaic virus protein is thus similar in certain respects to those of myosin and sodium thymonucleate.*

249431—40——7

The first experiments were concerned with the estimation of the viscosity as a function of pressure for the protein in water alone, in the presence of urea, and in guanidine HCl. The data are collected in table 3 and plotted in figure 6.

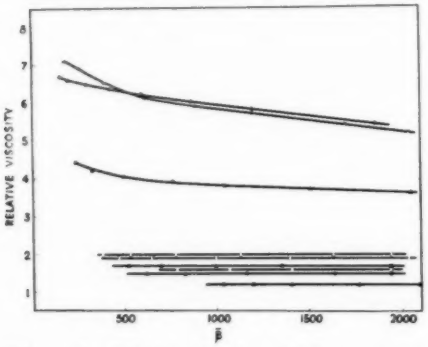



FIGURE 6.—Relative viscosity of 2.8-percent rat serum albumin in sodium thymonucleate (preparation No. 1) and salt mixtures as a function of the velocity gradient.

● Protein in water, ◆ in 8M urea, ■ in 8M guanidine HCl.

○ Protein plus 0.1 percent sodium thymonucleate in water, ◇ in 8M urea, □ in 8M guanidine HCl.

⊕ Protein plus 0.5 percent sodium thymonucleate in water, ⊕ in 8M urea, ⊠ in 8M guanidine HCl.

It is evident that the viscosity of the protein in water is independent of the external pressure applied, i. e., the solution follows the law of Poiseuille. Frampton (*13*) has reported the same phenomenon for this protein. Polson (*28*) has pointed out that the viscosity of a fairly asymmetric protein in water, namely, helix hemocyanin (axial ratio 16.7), is independent of the external pressure. It is probable that a protein molecule must possess an extremly high degree of asymmetry in order to show the phenomenon of structural viscosity. Myosin and also the tobacco mosaic virus protein, as Lauffer (*37*) and Frampton (*13*) have shown, fulfill this condition. The serum albumin molecule, from the independent evidence of Polson (*28*) and Neurath and Saum (*38*), has a comparatively low degree of asymmetry, and is with propriety classed with the "globular" proteins.

TABLE 3.—*The viscosity and streaming birefringence of rat serum ablumin in salts and in sodium thymonucleate*

[The concentration of serum albumin was always 2.8 percent. The pH in water and in the salts was 6.0. The pH in mixtures with sodium thymonucleate was 8.0. Temperature was 30° C.]

| Solvent | Salt | Concentration of salt | Specific viscosity [1] | Streaming birefringence |
|---|---|---|---|---|
| | | *mM added per cc. solution* | | |
| $H_2O$ | | | 0.21 | − |
| | Guanidine HCl | 1.0 | .25 | − |
| | | 8.0 | .73 | − |
| | Urea | 1.0 | .22 | − |
| | | 8.0 | .54 | − |
| 0.1 percent Na thymonucleate (preparation No. 1). | | | .66 | − |
| | Guanidine HCl | 8.0 | 0.92 | − |
| | Urea | 8.0 | 1.09 | − |
| 0.5 percent Na thymonucleate (preparation No. 1). | | | 4.02 | + |
| | Guanidine HCl | 8.0 | 2.67 | − |
| | Urea | 8.0 | 4.13 | + |

[1] Extrapolated from the viscosity-$\bar{\beta}$ curves for very high values of $\bar{\beta}$.

The viscosity of serum albumin in the presence of salts also follows the law of Poiseuille, but the order of magnitude of the viscosity is greatly changed (table 3, and fig. 6). In 1M urea and guanidine HCl, the viscosities of the solutions increase, the increase being slightly greater in the latter salt than in the former. The effect is more clearly revealed in the presence of 8M salt, for here the viscosity of the solutions very markedly increase, and again guanidine HCl increases the viscosity more than does urea. The relative effects on serum albumin of urea and guanidine HCl are consistent with the other criteria of denaturation employed, i. e., —SH groups appear in serum albumin dissolved in guanidine HCl but not in urea (*32–34*). Anson and Mirsky (*39*) showed that the

viscosity of the "globular" proteins are increased in urea, while Neurath and Saum (*38*) showed that $\nu$ for serum albumin increased from 6.50 in water to 22.6 in 6.66M urea. Recently Bull (*40*) demonstrated the marked rise of the viscosity of egg albumin in urea. Neurath and Saum and Bull suggested that this increase in viscosity is owing to a change in the shape of the protein molecule in the direction of greater asymmetry. Whatever the explanation may be, these data on serum albumin and egg albumin in certain salt solutions are in marked contrast with those of sodium thymonucleate and of proteins such as myosin and the tobacco mosaic virus. The viscosity of the albumins increases, the viscosity of thymonucleate, myosin, and tobacco mosaic virus protein decreases when the same salts are added to solutions of each. The relative order of effect is also curiously preserved, thus guanidine HCl, which raises the viscosity of serum albumin more than urea, also decreases the viscosity of myosin more than urea. Without necessarily assuming any interpretations, there is evidently a considerable difference in the behavior of certain "globular" proteins and certain fibrous substances such as thymonucleate toward the same salts. With this difference in mind, we investigated the behavior of mixtures of the two types of substances in the presence of these salts, with particular reference to protein and nucleic acid.

Nucleic acid was mixed in two different proportions with serum albumin, and the viscosity at different pressures determined in water alone, in the presence of 8M urea, and in the presence of 8M guanidine HCl. When the sodium thymonucleate was added to the isoelectric protein, a heavy precipitate of the mixture appeared. Addition of sodium hydroxide solution to pH 8.0 resulted in a clear solution, and measurements were necessarily performed under this condition. The thymonucleate was added in such amount that in one mixture it was about 3.6 percent of the protein, and in the other about 18 percent. If each component of the mixture reacts individually with the added salt, it would be expected that the effect of the salt in increasing the viscosity of the protein would be counterbalanced by the effect of decreasing the viscosity of the thymonucleate. The resultant viscosity would be a function of the algebraic sum of the two effects. The data are given in table 3, and the curves relating the viscosity-velocity gradient function are given in figure 4.

The first noticeable effect is that the value for the viscosity of the nucleic acid-protein mixture is between that of the thymonucleate and the protein considered separately (tables 1 and 3). The effect of the protein at 2.8-percent concentration (approximately $4 \times 10^{-4}$ molar) in diminishing the specific viscosity of 0.5-percent thymonucleate from 6.53 to 4.02 is very much greater than the decrease to 4.92 brought about by 1M glycine (tables 2 and 3). These and subsequent effects are much too large to be ascribed to the comparatively small effects caused by changes in pH. When urea and guanidine HCl are added to a mixture of 0.1-percent thymonucleate and protein there is a distinct rise in the viscosity of the mixture, but in contrast with the case of the protein alone, the viscosity in urea is greater than in guanidine HCl. The reason is obviously owing to the fact that whereas the salts simultaneously increase the viscosity of the protein and decrease the viscosity of the thymonucleate, the effect on the protein is greater than that on the thymonucleate. The relative increase in the viscosity of the mixture is greater in urea because the contribution

to the total viscosity owing to the thymonucleate is lowered more by guanidine HCl than by urea (table 2). When 0.5 percent thymonucleate is mixed with the protein, the relative decrease of the viscosity of the thymonucleate is smaller than when the ratio of protein to thymonucleate is higher. The contribution to the total viscosity of the mixture by the higher concentration of thymonucleate predominates; and thus when guanidine HCl is added, the specific viscosity of the mixture drops from 4.02 to 2.67. That the contribution of the protein is still appreciable is indicated by the effect of 8M urea, for in the presence of this salt the viscosity of the mixture increased slightly (table 3). In the latter case, even in the presence of a large amount of thymonucleate, 8M urea lowered the viscosity of the latter to a less extent than it increased the viscosity of the protein.[7]

Finally, the intensity of the streaming birefringence of 0.5-percent thymonucleate was diminished but still apparent in mixtures of the latter with protein in water and in 8M urea. In 8M guanidine HCl it disappeared.

It must be emphasized that the foregoing results were obtained on definite mixtures of protein and nucleic acid. Each component in these mixtures acts as a separate entity. The results obtained by adding urea and guanidine HCl to solutions of nucleoproteins derived from tissues will be presented in succeeding papers.

## SUMMARY

The relative viscosity of sodium thymonucleate as a function of the velocity gradient has been investigated in water, in salt solutions of various concentration, and in mixtures of proteins and salts. Estimations of the intensity of the double refraction of flow in these solutions have been made.

The viscosity of thymonucleate is a function of the velocity gradient, i. e., solutions of this substance possess anomalous or structural viscosity. The addition of salts results in a parallel decrease in the viscosity and in the intensity of birefringence. At sufficiently high concentrations of certain salts the structural character of the viscosity function as well as the double refraction of flow apparently disappear. The most effective anion in this respect is iodide, the most effective cation is guanidinium. The significance of the position of the latter group is discussed. The relative differences of various salts increase with the concentration of salt. Comparisons are drawn between the behavior of myosin and the tobacco mosaic virus protein.

The magnitude of the viscosity of sodium thymonucleate and the apparent intensity of the streaming birefringence depend upon the past history and the method of preparation. Preparations obtained by hot alkaline treatment of tissues possess these properties to a lesser degree than do those obtained by gentler means.

The remarkable reversibility of the salt effect in sodium thymonucleate is described. Solutions in the presence of salt which has apparently destroyed the structural viscosity and streaming birefringence have these properties almost completely restored by the removal of the salt.

The viscosity of rat serum albumin increases and that of sodium thymonucleate decreases, when the same salts are added to solutions of each. The viscosity of mixtures of protein and thymonucleate treated with these salts reveals the relative contribution to the total viscosity made by each of the components. Each component in the nucleic acid-protein mixture behaves like a separate entity.

[7] *The viscosity in solution of 5-percent thymonucleate in the presence of the protein is still structural (fig. 4).*

## REFERENCES


(*1*) HAMMARSTEN, E.: Edward Gamaliel Janeway lecture; function of thymonucleic acid in living cells. J. Mt. Sinai Hosp., **6:** 115–125 (1939).

(*2*) OPPENHEIMER, C., and STERN, K. G.: Biological oxidation. The Hague and New York (1940).

(*3*) VERWORN, M.: Studien zur Physiologie der Flimmerbewegung. Pflugers Arch. f. die Ges. Physiol., **48:** 149–180 (1890).

(*4*) LEVENE, P. A., and BASS, L. W.: Nucleic acids. New York (1931).

(*5*) THANNHAUSER, S. J., and ANGERMANN, M.: Experimentelle Studien über den Nucleinstoffwechsel. XXI. Mitteilung. Ueber die fermentative Aufspaltung der Thymusnucleinsäure mit Lebernucleotidase zur Darstellung von Purin- und Pyrimidinkohlehydratkomplexen. (Vorläufige Mitteilung). Ztschr. f. physiol. Chem., **186:** 13–25 (1929).

(*6*) FEULGEN, R.: Zur Darstellung der Nucleinsäure aus Kalbsthymus. Ztschr. f. physiol. Chem., **90:** 261–270.

(*7*) BREDERECK, H., and MULLER, G.: Ueber die Thyminsäure (Nucleinsäuren, XII. Mitteil.). Ber. d. deutsche chem. Ges., (A) **72:** 115–121 (1939).

(*8*) CASPERSSON, TORBJÖRN: Ueber den chemischen Aufbau der Strukturen des Zellkernes. Skandinav. Arch. f. Physiol., **73,** Suppl. No. 8, 154 pp. (1936).

(*9*) HAMMARSTEN, E.: Zur Kenntnis der biologischen Bedeutung der Nucleinsäureverbindungen. Biochem. Ztschr., **144:** 383–466 (1924).

(*10*) SIGNER, R., CASPERSSON, T., and HAMMARSTEN, E.: Molecular shape and size of thymonucleic acid. Nature, London, **141:** 122 (1938).

(*11*) Edsall, J. T.: Anisotropy. *In* Schmidt, C. L. A., editor, The chemistry of the Amino Acids and Proteins, pp. 527–539. Springfield, Ill., and Baltimore (1938).

(*12*) EDSALL, J. T., and MEHL, J. W.: The effect of denaturing agents on myosin. II. Viscosity and double refraction of flow. J. Biol. Chem., **133:** 409–429 (1940).

(*13*) FRAMPTON, V. L.: Viscosimetric studies on the tobacco mosaic virus protein. I. J. Biol. Chem., **129:** 233–244 (1939).

(*14*) SCHMIDT, G., and LEVENE, P. A.: Effect of nucleophosphatase on "native" and depolymerized thymonucleid acid. Science, **88:** 172–173 (1938).

(*15*) BINGHAM, E. C., and JACKSON, R. F.: Standard substances for the calibration of viscometers. U. S. Bur. Standards Scient. Paper No. **298** (1917).

(*16*) BINGHAM, E. C., and GREEN, H.: Paint, a plastic material and not a viscous liquid; the measure of its mobility and yield value. *In* Am. Soc. for Testing Materials Proc. Ann. Meeting **22,** vol. **19,** pt. 2, pp. 640–664 (1919).

(*17*) APPLEBY, M. P.: The viscosity of salt solutions. J. Chem Soc., **97:** 2000–2025 (1910).

(*18*) GETMAN, F. H.: A study of the solutions of some salts exhibiting negative viscosity. J. Am. Chem. Soc., **30:** 721–737 (1908).

(*19*) BURGERS, J. M.: Second report on viscosity and plasticity. Amsterdam (1938).

(*20*) EISENSCHITZ, R.: Die Viscosität von Suspensionen langgestreckter Teilchen und ihre Interpretation durch Raumbeanspruchung. Ztschr. f. physik. Chem., (A) **158:** 78–90 (1931).

(*21*) ——— Der Einfluss der BROWNSCHEN Bewegung auf die Viscosität von Suspensionen. Ztschr. f. physik. Chem., (A) **163:** 133–141 (1933).

(*22*) GUTH, E.: Untersuchungen über die Viskosität von Suspensionen und Lösungen. I. Ueber die Viskosität von Suspensionen. Kolloid Ztschr., **74:** 147–172 (1936).

(*23*) KUHN, W.: Ueber Teilchenform und Teilchengrosse aus Viscosität und Strömungsdoppelbrechung. Ztschr. f. physik. Chem., (A) **161:** 1 (1932).

(*24*) ——— Ueber quantitative Deutung der Viskosität und Strömungsdoppelbrechung von Suspensionen. Kolloid Ztschr., **62:** 269–285 (1933).

(*25*) PETERLIN, A.: Ueber die Viskosität von verdünnten Lösungen und Suspensionen in Abhängigkeit von der Teilchenform. Ztschr. f. Physik, **111:** 232–263 (1938).

(*26*) ——— Bestimmung der Grösse und des Achsenverhältnisses von ellipsoid-förmigen starren Teilchen aus der inneren Reibung von verdünnten Suspensionen. Kolloid Ztschr., **86:** 230–241 (1939).

(*27*) HATSCHEK, E.: Die Viskosität der Dispersoide. Kolloid Ztschr., **7:** 301–304 (1910).

(*28*) POLSON, A.: Ueber die Berechnung der Gestalt von Proteinmolekülen. Kolloid Ztschr., **88:** 51–61 (1939).

(*29*) KROEPELIN, H.: Untersuchungen an lyophilen Kolloiden, I. Kolloid Ztschr., **47:** 294–304 (1929).

(*30*) BOEDER, P.: Ueber Strömungsdoppelbrechung. Ztschr. f. Physik., **75:** 258–281 (1932).

(*31*) WILLIAMS, J. W., and CADY, L. C.: Molecular diffusion in solution. Chem. Rev., **14:** 186–217 (1934).

(*32*) GREENSTEIN, J. P.: Sulfhydryl groups in proteins. I. Egg albumin in solutions of urea, guanidine, and their derivatives. J. Biol. Chem., **125:** 501–513 (1938).

(*33*) ——— Sulfhydryl in proteins. II. Edestin, excelsin, and globin in solutions of guanidine hydrochloride, urea, and their derivatives. J. Biol. Chem., **128:** 233–240 (1939).

(*34*) ——— Sulfhydryl groups in proteins. III. The effect on egg albumin of various salts of guanidine. J. Biol. Chem., **130:** 519–526 (1939).

(*35*) GREENSTEIN, J. P., and EDSALL, J. T.: The effect of denaturing agents on myosin. I. Sulfhydryl groups as estimated by porphyrindin titration. J. Biol. Chem., **133:** 397–408 (1940).

(*36*) STANLEY, W. M., and LAUFFER, M. A.: Disintegration of tobacco mosaic virus in urea solutions. Science, **89:** 345–347 (1939).

(*37*) LAUFFER, M. A.: The viscosity of tobacco mosaic virus protein solutions. J. Biol. Chem., **126:** 443–453 (1938).

(*38*) NEURATH, H., and SAUM, A. M.: The denaturation of serum albumin. Diffusion and viscosity measurements of serum albumin in the presence of urea. J. Biol. Chem., **128:** 347–362 (1939).

(*39*) ANSON, M. L., and MIRSKY, A. E.: The effect of denaturation on the viscosity of protein systems. J. Gen. Physiol., **15:** 341–350 (1932).

(*40*) BULL, H. B.: Viscosity of solutions of denatured and of native egg albumin. J. Biol. Chem., **133:** 39–49 (1940).

# CHEMICAL STUDIES ON THE COMPONENTS OF NORMAL AND NEOPLASTIC TISSUES. II. THE NUCLEOPROTEIN FRACTION OF NORMAL ANIMAL LIVER

By JESSE P. GREENSTEIN, *research fellow*, with the technical assistance of WENDELL V. JENRETTE,
*National Cancer Institute, National Institute of Health, United States Public Health Service*

## INTRODUCTION

Although numerous studies of certain types of proteins have been made, there is a considerable lack of information on the proteins of mammalian tissues. With the exception of the proteins of the blood which have been widely studied, investigations of the animal proteins from several points of view have been chiefly confined to myosin (*1–5*), and to substances of specific function such as hormones (*6–8*), and enzymes (*9, 10*). There are recognizable difficulties associated with the investigation of the animal-tissue proteins, not the least of which are the relative unavailability of material, the presence of powerful proteases in certain tissues, and the pronounced lability of the proteins themselves when once removed from the tissue matrix. Some of these difficulties may be overcome for the greater part, if not entirely, by the proper choice of starting material and by the use of appropriate procedures. Among the latter may be mentioned rapidity in the working of the material, the invariable use of low temperatures, and the carrying on of as much of the procedure as is practicable at a pH relatively far removed from the optimum of the tissue protease.

With these factors in mind, it was decided to investigate the nucleoprotein fraction of normal mammalian liver. Several reasons other than the availability of the tissue led to this study: (1) The desire to investigate the proteins of liver tumors, several types of which are at present being studied in the National Cancer Institute. Before such an investigation could properly begin, however, it was necessary to have adequate information on comparable protein fractions of normal animals, and such information has hitherto been of the most fragmentary character; (2) the choice of the nucleoprotein fraction was decided upon because possible chemical alterations in the tissue during the cancer process might be reflected not only in the protein but also in the properties of the nucleic acid component; (3) many physiologically active proteins, such as certain plant, fowl, and animal viruses (*11–16*), including filterable cancer-producing agents, have been shown to be nucleoproteins; and (4) the choice of the nucleoprotein promised the practical advantage of working with a protein whose insolubility might permit a ready physical isolation from the soluble tissue proteases.

Several investigations on the fractionation of the proteins of liver have been reported. An account of the older literature is given in recent papers by Luck (*17, 18*). None of the liver proteins described in the older reports were characterized by anything more than their content of nitrogen and phosphorus and their coagulation temperatures.[1] Luck's careful work on the fractionation of the liver proteins by means of salts was primarily concerned with the estimation of the relative amount of each fraction during the course of dietary changes, and was not extended

[1] *This procedure was also characteristic of an isolated instance by Oswald (19) who described what he termed a "nucleoprotein" derived from human-liver carcinoma.*

to the chemical characterization of any one of the fractions.

We therefore considered it desirable, before investigating the protein composition of the livers of normal animals and of animals bearing liver tumors, to concentrate first on the nucleoprotein fraction of normal liver and to characterize it by as many criteria as are available. Because of the interest in the comparative biochemistry of different species we studied the liver nucleoprotein of the rabbit, calf, cow, and rat. These studies included the characterization of the various nucleoprotein preparations from the standpoints of elemental composition, amino acid content, denaturation, sulfur distribution, fat content, and viscosity behavior in the presence and absence of salts.

Moreover, in order to be able to state definitely that we were working with a nucleoprotein, it was considered necessary to isolate the nucleic acid attached to the purified protein. The nucleic acid so isolated was described in terms of its specific properties.

## MATERIALS AND METHODS

### THE PREPARATION AND PROPERTIES OF THE LIVER NUCLEOPROTEIN

The method for the preparation of nucleoprotein fractions from tissues has generally consisted in an alkaline extraction followed by precipitation of the nucleoproteins by the addition of acid (*20*). The possibility of contamination by other protein components of the tissue concerned, chiefly by globulins, is very likely. Frequently the extraction has been conducted in a highly alkaline medium which involves the risk of denaturing the proteins and splitting off the nucleic acid. We therefore modified the general preparative procedure (1) by the use of extraction solutions with a pH of 10.5 or less and (2) by performing all precipitations in the presence of 0.8M salt.

The livers were removed as soon as possible after the death of the animal and worked up immediately. All the work without exception was conducted in a cold room at a temperature not above 5° C. Four separate preparations were made of the rabbit nucleoprotein, and in each case the livers of about 10 to 14 animals were pooled and each batch worked up separately. Three of the four batches were derived from animals which carried the Brown-Pearce testicular tumor, the fourth batch was obtained from normal animals. A few metastases were found in the livers of 2 or 3 of the 35 rabbits which bore the tumor, and these were removed. The presence of the tumor apparently had no other effect on the liver. The gall bladder was also carefully dissected from each rabbit liver. The livers from the normal animals and 1 batch of livers from the testicular tumor-bearing animals were perfused with saline previous to excision.

No differences were found in the properties of the nucleoprotein preparations from perfused and nonperfused livers. It would be expected from the procedure described later that the proteins in the small amount of residual blood in the livers would not appear in the nucleoprotein fraction. This was confirmed by working up about 200 cc. of rabbit blood according to the procedure for obtaining liver nucleoprotein. No trace of protein was obtained from the blood when treated in this manner. It is true, however, that the nucleoprotein obtained from the perfused livers was slightly lighter in color than that from the nonperfused organs, but the minute amount of hemin breakdown products in the latter preparation imparted color but no appreciable weight to the protein. The necessity for perfusion may well be emphasized, nevertheless, if the albumin and globulin

fractions of the liver are to be investigated (*17*), for here the possibility of contamination with corresponding fractions of the residual blood is fairly certain.

In addition to the 4 rabbit preparations, 2 separate batches of rat-liver nucleoprotein were prepared. Each batch of rat protein represented the pooled livers of 50 animals. The rats were decapitated and exsanguinated. One batch of 50 livers was perfused with saline previous to excision, the other batch of 50 was not perfused. Again, no chemical difference between the 2 preparations was observed, with the exception that the nucleoprotein derived from the perfused livers was lighter in color than that from the nonperfused organs. Two separate preparations, each from 5 pounds of calf liver were also made, together with a single preparation from 5 pounds of cow liver.

The tissues in each case were finely minced in a meat chopper and then ground in a mortar with sand. In some cases 100 cc. of toluene was added during the rubbing with sand, but this was found to be unnecessary. The ground tissue was then suspended in five times the amount of a chilled solution of 0.5 M KCl and 0.03 M $NaHCO_3$ and stirred gently for 6 hours. The pH of the mixture was about 8.0. The extract was then filtered through gauze and treated with 50-percent acetic acid to a pH of 4.2 (bromcresol green). A few drops of caprylic acid stopped the foaming. A heavy precipitate of protein appeared. Early the next day the mixture was centrifuged, the supernatant liquid discarded, and the precipitate thoroughly washed with a 0.2 M acetate buffer at pH 4.2 containing 0.5 M KCl. The wash liquid was removed at the centrifuge and the residual protein stirred for 15 minutes with sufficient solution containing 0.5 M KCl plus 0.3 N $Na_2CO_3$ to yield a pH of 10.5. The mixture was centrifuged, and the residual, undissolved protein was discarded. The supernatant solution was then treated with 50-percent acetic acid to pH 4.2. In about 15 minutes the protein began to flocculate and after 1 hour had settled out. It was immediately centrifuged, the supernatant liquid discarded, and the protein precipitate thoroughly washed with the KCl-acetate buffer as described above. A small portion was removed and analyzed for nitrogen and phosphorus. After the washing, the main bulk of the protein precipitate was again suspended in the KCl-$Na_2CO_3$ solution and stirred for 15 minutes and centrifuged. This time there was only a minute amount of undissolved protein. The supernatant was acidified again to pH 4.2, and the precipitate centrifuged and washed as before. The process of precipitating, washing, and redissolving was repeated several times more, 6 in all, and at each precipitation a small amount of protein was removed and analyzed for nitrogen and phosphorus. After the second precipitation, the $\frac{N}{P}$ ratio for successive precipitations was practically constant for every preparation and amounted to $15.0 \pm 0.2$. Comparatively little protein was lost after the second precipitation in spite of the several successive manipulations, but nevertheless there was always a slight amount of denatured protein at each step which had to be discarded. All the preparations derived from the different species of animals behaved alike during the course of the procedure. After the final step, each preparation was thoroughly washed several times at the centrifuge with water containing a few drops of acetic acid until the salt was removed, and then dried at very low temperature under vacuo in the lyophilizing apparatus of Flosdorf-Mudd. The preparation after this treatment was

a light tan, nonhygroscopic powder, completely free of glycogen.[2]

The protein when freshly precipitated is soluble at pH values above 8 in solution which may or may not contain salt. The solutions are very turbid and milky, clarity never being achieved even at very high alkalinities. Clear, transparent solutions are, however, obtained readily by adding powdered guanidine HCl or other guanidine salts, but not by adding urea. (See below.) The rate of spontaneous denaturation in aqueous solutions at pH 8 is fairly rapid, and work performed on solutions of the protein in water must be accomplished quickly.

It was expected that the method of preparing the nucleoprotein fraction, by employing salt solutions at every step of precipitation, washing, and solution might remove not only the albumins but also the globulins of the liver. It was fortunate that the nucleoprotein is insoluble in salt at pH 4.2. That the nucleoprotein obtained was very largely, if not completely, free of other protein components of the liver is suggested not only by the procedure adopted and by the constancy of the $\frac{N}{P}$ ratio, but also by the fact that no preparation after drying amounted to more than 1 percent of the total wet weight of the liver batch used. Since the total protein content of the liver varies from 15 to 25 percent, the nucleoprotein represents only a small fraction of the total protein content. Indeed, if one collects the supernatant and the washings from the first and second precipitations and adds ammonium sulfate to saturation, a very heavy precipitate of protein occurs, which may presumably be the albumin and globulin components.

[2] *Mucins were also apparently absent from the preparations, since on boiling with 2 N HCl for 3 hours no trace of sulfate was found in the digest.*

As previously mentioned, all the work must be conducted at about 5° C. and every solution thoroughly chilled before use. The centrifuge itself was installed in the cold room. Another point is the need for rapid working. In no case was the total length of time from the excision of the livers to the final precipitation and washing greater than 3 days. A batch of minced rabbit liver allowed to stand in a solution at pH 7 for 2 weeks in the cold room yielded only a minute amount of the nucleoprotein. The $\frac{N}{P}$ ratio of the protein was very nearly the same as that of much larger yields obtained by more careful and rapid treatment. No further investigation of this particular protein could be made because of lack of material. The decrease in yield in this instance is presumably owing to the action of liver cathepsin. It is possible that even the larger yields obtained according to the more rapid procedure are actually lower than they should be because of this factor, but it is difficult, if not impossible, to prevent this loss if it does occur. The primary extraction with $KCl–NaHCO_3$ at pH 8 was chosen so as to reduce the protease action by working at a pH far removed from the optimum of the lytic action of cathepsin (*21*).

## THE USE OF THE TERM "NUCLEOPROTEIN"

The reproducibility of the various preparations is so good (tables 1 and 2) as to encourage the feeling that one is dealing here with a protein fraction of apparently constant properties.[3] On the other hand, no claim is made that we are

[3] *Kelley (23) has shown that nucleoproteins of approximately constant composition may be obtained from various animal tumors. On the other hand, Heidelberger and Kendall (24) have shown that the nucleoproteins derived from certain bacteria may be themselves separated into various fractions by altering the pH of their solutions.*

TABLE 1.—*Analytical values for the liver nucleoprotein preparations*

[Preparations previously treated with hot alcohol-acetone-ether, and dried subsequently at room temperature]

| Source | Preparation No. | N | Amide N | P | Total S | Free—SH in native protein as cysteine | Free—SH in denatured protein as cysteine [6] | Cystine-cysteine | Methionine | Tyrosine | Tryptophane |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* |
| Rabbit [1] | 1 | 15.6 | 1.0 | 0.75 | 1.04 | 1.3 | 1.3 | 1.3 | 3.0 | 3.8 | 1.3 |
| Do.[2] | 2 | 15.6 | 1.0 | .80 | 1.18 | 1.4 | 1.4 | 1.3 | 3.1 | 3.9 | 1.3 |
| Do.[3] | 3 | 15.6 | .9 | .80 | 1.18 | 1.4 | 1.4 | 1.4 | 2.9 | 4.0 | 1.5 |
| Do.[3] | 4 | 15.7 | 1.0 | .79 | 1.13 | 1.2 | 1.2 | 1.5 | 3.1 | 4.0 | 1.5 |
| Calf | 5 | 15.6 | 1.0 | .82 | 1.09 | .7 | .7 | 1.4 | 3.2 | 3.9 | 1.4 |
| Do | 6 | 15.7 | 1.0 | .80 | 1.14 | .7 | .7 | 1.4 | 3.1 | 3.9 | 1.5 |
| Rat [4] | 7 | 15.6 | .9 | .85 | 1.17 | .2 | .2 | 1.3 | 3.0 | 3.8 | 1.5 |
| Do.[5] | 8 | 15.7 | 1.0 | .80 | 1.22 | .2 | .2 | 1.4 | 3.1 | 3.8 | 1.5 |
| Cow | 9 | 15.8 | 1.0 | .85 | 1.15 | .6 | .6 | 1.5 | 3.1 | 3.7 | 1.7 |

[1] Livers obtained from normal animals and perfused with saline before excision.
[2] Livers obtained from animals with Brown-Pearce testicular tumors and perfused with saline before excision.
[3] Livers obtained from animals with Brown-Pearce testicular tumors and not perfused before excision.
[4] Livers perfused before excision.
[5] Livers not perfused before excision.
[6] Denatured in 10M guanidine HCl solution.

dealing here with a single chemical individual. It is regarded as a protein complex of approximately constant composition. To avoid cumbersome terminology, the word "fraction" is generally omitted from the text, but this qualification must always be borne in mind. From recent evidence it is doubtful if any protein so far isolated may be considered as a chemical individual (*22*), and certainly it would be surprising if a nucleoprotein derived from complex glandular tissue would not itself be complex in composition.

## DISCUSSION

### NITROGEN AND PHOSPHORUS

Portions of the various protein preparations after drying in the lyophilizing apparatus were decomposed in concentrated $H_2SO_4$ and the nitrogen determined by the direct nesslerization procedure of Koch and McMeekin (*25*). The average nitrogen value estimated was about 11 to 13 percent and was obviously too low. It was thought that the possibility of incomplete drying (*26*) might provide one explanation for this, and so the various samples were boiled for one-half hour with a mixture of equal parts of alcohol and acetone. The dried protein after this procedure yielded nitrogen values in the neighborhood of 15.8 percent. When the alcohol-acetone extracts were evaporated to dryness, a quantity of fat was discovered which accounted almost entirely for the difference in the nitrogen values. The study of the

TABLE 2.—*Sulfur distribution of the liver nucleoprotein preparations*

[Values given on a lipoid-free, dry-weight basis for each protein]

| Source | Total S | Cysteine S in intact protein | Cystine-cysteine S | Methionine S | Ratio, $\frac{\text{Cystine-cysteine S+methionine S}}{\text{Total S}}$ |
|---|---|---|---|---|---|
| | *Percent* | *Percent* | *Percent* | *Precent* | |
| Rabbit No. 1 | 1.04 | 0.35 | 0.34 | 0.65 | 0.95 |
| Rabbit No. 2 | 1.18 | .27 | .34 | .67 | .86 |
| Rabbit No. 3 | 1.18 | .37 | .37 | .62 | .84 |
| Rabbit No. 4 | 1.13 | .32 | .40 | .67 | .95 |
| Calf No. 5 | 1.09 | .19 | .37 | .69 | .97 |
| Calf No. 6 | 1.14 | .19 | .37 | .67 | .91 |
| Rat No. 7 | 1.17 | .05 | .34 | .65 | .85 |
| Rat No. 8 | 1.22 | .05 | .37 | .67 | .85 |
| Cow No. 9 | 1.15 | .16 | .40 | .67 | .93 |

fat associated with the nucleoprotein will be discussed later. It is sufficient to state here that for the final estimation of nitrogen, the proteins were exhaustively extracted with the boiling alcohol-acetone mixture and then washed several times on the funnel with ether. The dried protein served not only for the analyses of nitrogen and phosphorus by the Fiske-Subbarow method, but also for every other estimation save —SH. The pertinent data for nitrogen and phosphorus are in table 1.

The nitrogen values for the various preparations are $15.8 \pm 0.2$ percent. Phosphorus values are all close to 0.8 percent. The concordance of these values for all the preparations is striking.

### AMIDE NITROGEN

For the estimation of amide nitrogen, 300 mg. of each preparation was boiled for 2 hours in 2N HCl under a reflux condenser. The solutions were then transferred to a distilling flask, a few boiling chips added, and the solutions treated with 5N NaOH until distinctly alkaline. The solutions were boiled for 15 minutes, and the distillate was trapped in 0.5N $H_2SO_4$. Estimations of ammonia in the latter solution were made by direct nesslerization (*25*). The data for amide nitrogen are also shown in table 1. The values for the various preparations are very nearly identical and amount to about 6.3 percent of the total N for each preparation.

### TOTAL SULFUR

The method of analysis for sulfur consisted in an alkaline-peroxide fusion. Five hundred mg. of the protein in a nickel crucible of 150-cc. capacity was treated with 15 cc. of 40-percent NaOH, and the mixture boiled gently and carefully over a microburner for 10 minutes. The resulting solution after cooling was carefully treated, drop by drop, with 0.5 cc. of 30-percent $H_2O_2$, and then boiled for several minutes, care being taken to avoid too much foaming. When the excess peroxide was boiled off, the mixture was allowed to cool, and again peroxide was added. On reheating less foaming was encountered, and the mixture was boiled for a longer interval. More peroxide was subsequently added and the mixture kept in a gently boiling state until nearly every trace of carbonaceous material had disappeared. The total amount of peroxide necessary was usually 10 cc., and the length of time for the entire procedure was about 30 minutes. The final oxidized digest was cooled, dissolved in 200 cc. of distilled water and treated with 10-percent HCl until just acid to Congo red. The solution was filtered, and the filtrate brought to 80° C. and then treated with 10 cc. of 10-percent $BaCl_2$ solution. After standing overnight, the precipitated $BaSO_4$ was filtered on sintered glass funnels, dried, and weighed.

The data for total S are given in table 1, and like the analyses described above, are remarkably similar for all the preparations, regardless of the species of animal.

### TYROSINE AND TRYPTOPHANE

The amino acids, tyrosine and tryptophane, were estimated in the various protein preparations after a 24-hour digestion in 20-percent NaOH solution. The method of estimation followed the excellent procedure of Lugg (*27*, *28*), which permits the mercuration of the tyrosine and precipitation of the tryptophane in one step. The data are collected in table 1, and here also the remarkable concordance of the values for all the preparations is apparent. The tyrosine figures for the nine preparations are $3.8 \pm 0.2$ percent, and the tryptophane $1.5 \pm 0.2$ percent.

### CYSTINE-CYSTEINE AND METHIONINE

Methionine was estimated by the Baernstein procedure (*29*) which is based on the opening of the homocysteine thiolactone in the presence of strong ammonia solution. No attempt was made to use the corresponding volatile iodide method principally because the proteins had been previously treated with hot alcohol. The errors in the volatile iodide method when alcohol is used for drying the protein have been pointed out by Bailey (*30*). The results for the analyses for methionine are given in table 1, and amount, for the nine preparations, to about $3.1 \pm 0.1$ percent. The remarkable similarity in all preparations is notable.

Cystine-cysteine was estimated by the Baernstein method in concentrated HI. The results were $1.4 \pm 0.1$ percent for the nine preparations.

### —SH GROUPS AND DENATURATION

Solutions of each of the nucleoprotein preparations in 0.5 N KCl and at pH 8.0 gave a positive nitroprusside reaction. These solutions also reduced a certain amount of added porphyrindin dye. The presence of free —SH groups in the preparations was thereby indicated.[4]

These free sulfhydryl groups were estimated for each of the liver nucleoprotein preparations. The freshly precipitated protein in each case was dissolved in a $KCl\text{-}NaHCO_3$ solution at pH 8.0 and any denatured protein removed by centrifugation. The solutions were made up at 2.2–2.8 percent concentration of protein, and the free —SH groups were estimated by the porphyrindin technique described in detail recently by Greenstein and Edsall (*4*). The amount of porphyrindin reduced is expressed, as usual, in terms of its equivalent of cysteine per 100 gm. of protein, and the results are given in table 1. It is clear that the results for each preparation of a given species are fairly consistent, but that the absolute values for each species are quite different. The rabbit preparations show —SH groups equivalent to 1.3 percent cysteine, the rat preparations 0.2 percent, and the calf and cow 0.7 percent. There is no question of experimental error accounting for such differences—the latter must be due to inherent differences in the species from which the preparations were derived. These represent the first differences based on analytical determinations so far made on the preparations.

A great many proteins, whether they possess free —SH groups when in the native state or not, demonstrate an increase in the number of such groups when they are denatured. (See Greenstein (*31–33*) for more complete discussion.) To confine the discussion to proteins such as myosin, urease, and the liver nucleoproteins in the present paper, it may be said that the number of free —SH groups found in solutions of the former two proteins in the native state are greatly increased when the protein is denatured. The most effective denaturing agent is guanidine HCl (*4, 31–33*). When this reagent is added in 10M concentration to solutions of the liver nucleoproteins, a startling transformation takes place, for the originally turbid, milky solutions become transparent and fluid. Guanidine HCl is simply a neutral salt; whatever takes place in the protein solution does not presumably represent a chemical reaction but appears to be owing to some effect on the state of aggregation of the protein molecules. Such effects have been demonstrated with this and analogous substances (*5, 34*).

[4] *By the term "free sulfhydryl" is meant the fact that these groups are observable by the criteria of the nitroprusside and the porphyrindin reactions.*

When the —SH groups in the liver nucleoproteins dissolved in 10M guanidine HCl solutions were estimated, it was discovered that no increase in the number of such groups occurred. The proportion of titratable —SH groups after denaturation in guanidine HCl was the same as that found in the native protein. This point was checked further by adding porphyrindin in sufficient quantity to solutions of the native protein to oxidize completely the free —SH groups. When guanidine HCl was added and the solutions tested for the presence of —SH groups, none was apparent. The lack of increase in the —SH groups following denaturation with guanidine HCl may be accounted for in one of two ways: Either this salt is ineffective in completely denaturing the protein, or else there are no more —SH groups to be revealed, i. e., all the —SH groups which the protein possesses appear in the native state of the protein and none are masked and waiting to be released as a result of denaturation. No definite answer to either of these alternatives is available at present. Attempts to denature the proteins by use of heat, urea, or trichloracetic acid precipitation yielded no evidence of an increase in sulfhydryl groups. The fact that guanidine HCl was by far the most powerful denaturing agent on all the proteins hitherto investigated makes us inclined to the second of the above-mentioned alternatives. In any case, the results on denaturation with the liver nucleoproteins are in contrast with those obtained with myosin and with urease insofar as the sulfhydryl groups are concerned. The failure to show additional —SH groups on denaturation is common to all of the nucleoprotein preparations irrespective of the species from which the preparations are derived.

One further interesting point of comparison of the liver nucleoproteins with myosin may be illustrated. Greenstein and Edsall (*4*) have shown that the addition of very small amounts of substances containing the charged nitrogen atom, $N^+$, to native myosin results in some intramolecular change within this protein in such a fashion that the free —SH groups vanish. When substances of this character, such as glycine and ammonium chloride, were added even to saturation of the liver nucleoprotein, there was no such loss of the —SH groups. Apparently, this unexpected effect of $N^+$ is specific for myosin.

The data for the —SH groups expressed as cysteine per 100 gm. of protein are given in table 1.

### SULFUR DISTRIBUTION

Table 2 gives the data for the analyses of total sulfur, methionine sulfur, cystine-cysteine sulfur (Baernstein), and the sulfur of the cysteine revealed in the intact protein through the porphyrindin titration (table 1). Since the values of the latter are the same in the native and in the denatured preparations, no special designation is needed in table 2 to distinguish them.

It is clear that the sum of the cystine-cysteine sulfur and the methionine sulfur is very close to the value for the total sulfur of each preparation. In the rabbit preparations, the cysteine determined in the intact denatured protein is equal to the amount found in the hydrolyzed protein. In the calf, cow, and rat preparations, however, the amount of cysteine found in the intact protein is only a fraction of that found in the hydrolyzed protein. This difference may be tentatively interpreted as being due to the absence of cysteine in the rabbit preparation and its presence in the other preparations. In any event, the point is worth emphasizing that *so far as we have gone*

*in the study of the nucleoprotein fraction from different species, the only distinct chemical difference which we have observed among the various preparations has been in the proportion of the titratable —SH groups in the intact preparations and which we have designated in terms of cysteine. The difference in the proportion of this constituent from one species to another is far outside the possibility of experimental error, and this fact in conjunction with the obvious consistency of the results for each particular species leads us to believe that the differences found are real.*

The values for N, amide N, P, total S, cystine-cysteine, methionine, tyrosine, and tryptophane (table 1) are very nearly the same for all the preparations studied. The concordance of these values for the different species is similar to results of Bailey (*2*) on the myosins of different species. Bailey showed that the values for each of the above-mentioned constituents were very nearly the same, regardless of the species from which the myosin had been derived.

### FAT

It was stated above that the hot alcohol-acetone extract of the nucleoprotein preparations when evaporated to dryness yielded a considerable amount of fatty material. It was considered desirable to investigate further the fat apparently associated with the protein.

The preparations of protein used in this study were finally precipitated from solution, washed thoroughly with water to remove salt, and then dried in the lyophilizing apparatus at low temperature and at high vacuo. Samples of each preparation were then divided in half. One half was extracted with cold ether in a Soxhlet apparatus, the other half was extracted with a boiling mixture of equal parts of alcohol-acetone for 30 minutes, cooled, and extracted on the filter several times with ether. The total extracts by either procedure were evaporated separately to dryness at room temperature and to constant weight. The proportion of fatty material from each preparation obtained by both procedures is given in table 3.

TABLE 3.—*The proportion of fat in the liver nucleoprotein preparations*

| Source [1] | Preparation No. | Cold ether extract | Hot alcohol-acetone-ether extract | Fat from hot alcohol-acetone-ether extract | | P/N ratio |
|---|---|---|---|---|---|---|
| | | | | N | P | |
| | | *Percent* | *Percent* | *Percent* | *Percent* | |
| Rabbit | 1 | 4.4 | 10.1 | 0.7 | 1.6 | 2.3 |
| Do | 2 | 4.5 | 12.5 | .6 | 1.6 | 2.6 |
| Do | 3 | 4.0 | 9.6 | .7 | 1.9 | 2.7 |
| Do | 4 | 3.9 | 12.5 | .7 | 2.2 | 3.1 |
| Calf | 5 | 5.1 | 13.1 | .7 | 1.9 | 2.7 |
| Do | 6 | 16.3 | 21.5 | .6 | 1.5 | 2.5 |
| Rat | 7 | 5.5 | 14.7 | .8 | 2.1 | 2.6 |
| Do | 8 | 5.3 | 15.0 | .8 | 2.1 | 2.6 |
| Cow | 9 | 5.8 | 15.3 | .8 | 2.2 | 2.7 |

[1] See description of each preparation in table 1.

[2] The $\frac{P}{N}$ ratio for lecithin is 2.8.

It is apparent that, with the sole exception of one of the preparations from calf liver (No. 6), the proportion of ether-soluble fat and hot alcohol-acetone-ether fat is of the same order of magnitude for all of the preparations studied. If one excepts preparation No. 6, the proportion of the former ranges from 3.9 to 5.8 percent, and of the latter from 9.6 to 15.3 percent. The reason for the exceptional values for calf preparation No. 6 is not apparent.

In any event it is clear that the fat is present in these preparations in two types of association. One is easily removable with cold ether, the other requires a more vigorous extraction procedure. The proportion of fat found by the latter procedure and given in table 3 naturally includes the fat otherwise yielded by the former. The amount of the more firmly bound fat is one to two times more than that of the loosely bound fat removable with cold ether. A considerable proportion of the total fat, therefore, seems to be intimately

bound in the whole nucleoprotein complex, and one is reminded of the fat often associated with other types of nucleoproteins such as the vaccinia virus (*16*) and the Rous chicken sarcoma virus (*15*).

Both firmly and loosely bound yields of fatty material from the various liver nucleoprotein preparations showed the presence of nitrogen and phosphorus although the proportion of these elements was analytically determined only in the former. The values are given in table 3. The ratios of $\frac{P}{N}$ are of the same order of magnitude for all the preparations studied, including No. 6, and range from 2.3 to 3.1. This would indicate the possible presence of phospholipids, since the $\frac{P}{N}$ ratio for lecithin is approximately 2.8. The absolute values of P and N for the several preparations (including No. 6), indicate that the amount of phospholipid might be as much as 40 percent if all the N and P which were found belonged to phospholipid.

### ISOLATION AND CHARACTERIZATION OF THE NUCLEIC ACID COMPONENT OF COW LIVER NUCLEOPROTEIN

In order to be able to state definitely that we were working with nucleoproteins it was considered desirable to isolate the nucleic acid from the protein and to characterize it adequately. In the main, the purpose was one of identification. Cow-liver protein was chosen because large amounts of cow liver were available.

The protein was worked up as previously described from 5 pounds of fresh cow liver. The moist protein was treated with 1 liter of 5N NaOH and 200 gm. of sodium acetate and the mixture heated in a boiling water bath for 1 hour. At the end of this time the dark-brown-colored solution was treated with glacial acetic acid until a pH of 5.5–6.0 was reached. The denatured protein was filtered off on a hot-water funnel, and to the light-yellow-colored filtrate 2.5 times the volume of 95-percent alcohol was added, and the mixture allowed to stand over night. The nucleic acid, which appeared in a stringy, white mass, was filtered off, washed with dilute alcohol, and dissolved in a little hot water. The hot solution was filtered and the filtrate treated with a few crystals of sodium acetate and an equal volume of alcohol. This process was repeated once. The sodium nucleate finally obtained was dried with alcohol and ether and ground in a mortar to a fine white powder. The yield was 3.2 gm.

The nucleate gave strongly positive Diesche and Feulgen reactions for desoxypentose. No glycogen was present. Analyses for N and P yielded 14.5 and 8.9 percent, respectively. A portion was hydrolyzed in boiling methyl alcohol-HCl, and the crude adenine and guanine were isolated. The adenine amounted to 9.9 percent, the guanine to 10.5 percent. The nucleic acid was therefore well characterized from the analytical viewpoint. Small amounts of moisture and protein were present in the preparation.

The sodium nucleate thus obtained from cow-liver nucleoprotein bore further resemblance to the sodium nucleate isolated from calf thymus by the method of Levene (*35*). Solutions of this substance in water were quite viscous and showed streaming birefringence at sufficiently high concentration.

Measurements were made of the viscosity under varying velocity gradients and in the presence of salts (*35*). The viscosity-velocity gradient curves are given in figure 1. The data are collected in table 4. Comparison of $\nu_\infty$ in water with those pertaining to calf thymonucleate (*35*, *table 1*) indicates that the former is of a very much smaller order of magnitude. It is

true, of course, that the cow-liver protein nucleate and the calf thymonucleate were prepared by entirely different procedures. The former preparation showed double refraction of flow in 2-percent concentration. Addition of 8M NaI, urea, and guanidine HCl reduced the values of $\nu_\infty$ and apparently destroyed the streaming birefringence. In these respects the cow and the calf nucleate preparations are similar. The unusual effect of guanidine HCl on the viscosity—velocity gradient curve (fig. 1) is noteworthy. This particular curve was rechecked, and the same results were duplicated. It is possible that the shape of the curve is due to the effect of the guanidine HCl on the protein impurity in the preparation. The slight anomaly in the viscosity curve (fig. 1) of the nucleic acid in water is apparently exaggerated in the presence of guanidine HCl. In the presence of NaI the anomaly has completely disappeared.

TABLE 4.—*The viscosity and streaming birefringence of sodium nucleate from cow-liver nucleoprotein in water and in salts*

[Temperature 30° C.]

| Concentration of of Na nucleate (percent) | Salt | Concentration of salt | Specific viscosity [1] | $\nu_\infty$ | Streaming birefringence |
|---|---|---|---|---|---|
| | | *mM added per cc. of solution* | | | |
| 0.5 | | | 2.91 | 939 | − |
| 2.0 | | | 11.70 | 943 | + |
| 2.0 | NaI | 8.0 | 1.54 | 124 | − |
| 2.0 | Guanidine HCl | 8.0 | 3.43 | 277 | − |
| 2.0 | Urea | 8.0 | 6.06 | 488 | − |

[1] Extrapolated from the viscosity-$\overline{\beta}$ curves for very high values of $\overline{\beta}$.

In view of the identification of the substance investigated as nucleic acid, the protein fraction of liver which we have isolated may with propriety be referred to as "nucleoprotein fraction."

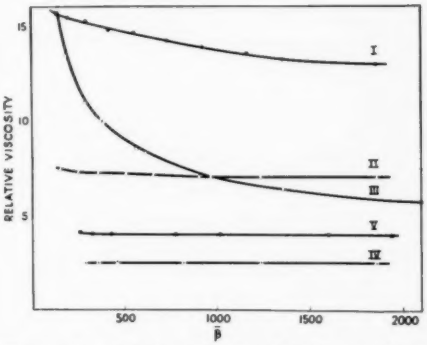



FIGURE 1.—Relative viscosity of sodium nucleate from cow-liver nucleoprotein as a function of the velocity gradient: I, 2-percent concentration in water; II, in 8M urea; III, in 8M guanidine HCl; IV, in 8M NaI; V, 0.5-percent concentration in water.

## VISCOMETRIC STUDIES OF COW-LIVER NUCLEOPROTEIN

Studies on the nucleoprotein of cow liver were conducted in the viscometer similar to those previously reported (*35*). The results with this protein are shown in table 5, and the viscosity-pressure gradient curves are given in figure 2. It is apparent

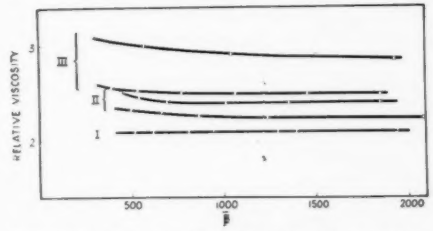



FIGURE 2.—Relative viscosity of cow-liver nucleoprotein, 2.2 percent, and pH 8.0, as a function of the velocity gradient. I, in water; II, in 4M salt; III, in 8M salt. ◇ Urea, □ Guanidine HCl.

that solutions of this protein follow Poiseuille's law, and that the addition of salts results not in a decrease in viscosity as occurs with nucleic acid, but in an increase in viscosity such as occurs with the

albumins (*35*). The behavior of this protein is identical with that of serum albumin, but not with that of a serum albumin-sodium nucleate mixture (*35*). In the latter mixture when the protein-nucleic acid ratio is of the same order of magnitude as that found in the liver nucleoprotein, the addition of salts results in an increase in the viscosity of the serum albumin-nucleate mixture. However, in this case urea increases the viscosity more than does guanidine HCl. The reasons for this have been discussed (*35*). On the other hand, in the cases of serum albumin and the liver nucleoprotein, guanidine HCl raises the viscosity of solution of these proteins more than does urea.

TABLE 5.—*The viscosity of nucleoproteins in water and in salts*

[The concentration of cow-liver nucleoprotein was 2.2 percent and pH 8.0. The concentration of calf-thymus nucleoprotein was 3.9 percent and pH 12.5. Temperature 30° C.]

| Source of protein | Salt | Concentration of salt | Specific viscosity [1] | $\nu_\infty$ |
|---|---|---|---|---|
| | | *mM added per cc. of solution* | | |
| Cow liver | | | 1.12 | 72 |
| | Guanidine HCl | 4.0 | 1.41 | 91 |
| | Urea | 4.0 | 1.28 | 83 |
| | Guanidine HCl | 8.0 | 1.82 | 118 |
| | Urea | 8.0 | 1.50 | 97 |
| Calf thymus | | | 14.07 | 515 |
| | Guanidine HCl | 4.0 | 3.34 | 122 |
| | Urea | 4.0 | 12.63 | 462 |
| | Guanidine HCl | 8.0 | 2.97 | 108 |
| | Urea | 8.0 | 11.42 | 418 |

[1] Extrapolated from the viscosity-$\bar{\beta}$ curves for very high values of $\bar{\beta}$.

It was considered desirable to make a further comparison with another nucleoprotein. A preparation from calf thymus which is high in associated nucleic acid was therefore chosen. The protein is soluble in fairly strong alkali at a pH of 12–13. The results are given in table 5, and the viscosity-velocity gradient curves in figure 3. These curves indicate that there is a slight but unmistakable anomaly

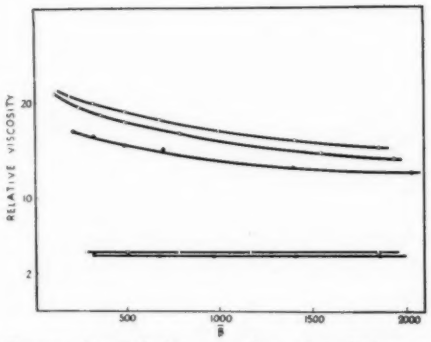



FIGURE 3.—Relative viscosity of calf-thymus nucleoprotein, 3.9 percent, and pH 12.5, as a function of the velocity gradient. ○ in water, ◇ in 4M urea, □ in 4M guanidine HCl, ◆ in 8 M urea, ■ in 8 M guanidine HCl.

in the viscosity in water. Addition of salts results in a decrease in the viscosity, guanidine HCl being more effective than urea. The results on this nucleoprotein are quite different from those on the liver nucleoprotein, the albumins, or serum albumin mixtures with small amounts of nucleic acid (*35*), and are more nearly like those described for serum albumin mixtures with a high proportion of nucleic acid (*35*). This might suggest that calf-thymus nucleoprotein is actually a mixture rather than a compound of protein and nucleic acid, but it must be remembered that measurements were made on this protein at a sufficiently high pH to hydrolyze effectively any primary chemical linkage of an ester type between the protein and nucleic acid components.

On the other hand, it appears that the liver nucleoprotein is not a simple mixture of protein and nucleic acid, but more probably a definite compound.[5] Some preliminary experiments involving attempts to separate the nucleic acid from the liver nucleoprotein by gentler means

[5] *Heidelberger and Scherp (36) have shown that the nucleic acid attached to the proteins of certain bacteria are bound more firmly than in dissociable salt linkage.*

than alkali, i. e., by denaturation with urea in neutral solution followed by dialysis, have failed so far. This problem is at present being investigated.

It may be mentioned that solutions of the liver nucleoprotein in water showed no streaming birefringence. Thus, not only in this property but also in that of viscosity, this protein is quite different from the tobacco mosaic virus and the chicken sarcoma agent which are also nucleoproteins.

## SUMMARY

The preparation of the nucleoprotein fraction from the livers of rabbits, rats, calves, and cows is described. The essential steps in the procedure involve precipitation at pH 4.2 in the presence of 0.8M salt, followed by washing of the precipitate by a KCl-acetate buffer.

Analytical data on all the nucleoprotein preparations are described. These include estimations for total N, amide N, P, total S, cystine-cysteine, methionine, tyrosine, and tryptophane. The values for each of these constituents appear to be very nearly the same for all the protein preparations regardless of the species from which the latter were derived.

The —SH groups in the native protein preparations were estimated by porphyrindin titration and were expressed in terms of cysteine per 100 gm. of protein. The values were very different for the protein preparations of each species. When the proteins were denatured by addition of guanidine HCl, there was no change in the proportion of these —SH groups.

The sum of the cystine-cysteine and the methionine sulfur is very nearly equal to the total sulfur in each preparation.

Comparisons are made of the analytical data on the liver nucleoproteins with those of myosin.

Each preparation contained from 10 to 15 percent fatty material, a high proportion of which appeared to be phospholipid. Part of this fat was removable by cold ether, the remainder required hot alcohol-acetone for removal from the protein.

From one of the protein preparations (cow liver) nucleic acid was isolated by a process involving warm alkaline hydrolysis. The nucleic acid thus obtained was characterized by means of analyses for N, P, adenine, guanine, and by specific color reactions for desoxypentose. The nucleic acid was further characterized by viscosity and streaming birefringence studies. The resemblance to thymonucleic acid from calf thymus is discussed.

Viscosity determinations under varying velocity gradients in the presence of different salts are reported for a preparation of cow-liver nucleoprotein and for a preparation of calf-thymus nucleoprotein. The results on the liver nucleoprotein suggest that it is not a mixture but a compound of protein with nucleic acid.

## REFERENCES

(*1*) WEBER, H. H.: Die Muskeleiweisskörper und der Feinbau des Skeletmuskels. Ergebn. d. Physiol., **36:** 109–111 (1934).

(*2*) BAILEY, K.: Composition of myosins and myogen of skeletal muscle. Biochem. J., **31:** 1406–1413 (1937).

(*3*) MIRSKY, A. E.: Protein denaturation. *In* Cold Spring Harbor Symposia on Quantitative Biology, **6:** 150–163 (1938).

(*4*) GREENSTEIN, J. P., and EDSALL, J. T.: The effect of denaturing agents on myosin. I. Sulfhydryl groups as estimated by porphyrindin titration. J. Biol. Chem., **133:** 397–408 (1940).

(*5*) EDSALL, J. T., and MEHL, J. W.: The effect of denaturing agents on myosin. II. Viscosity and double refraction of flow. J. Biol. Chem., **133:** 409–429 (1940).

(6) FRAENKEL-CONRAT, H., SIMPSON, M. E., and EVANS, H. M.: The effect of cysteine on gonadotropic hormones. J. Biol. Chem., **130:** 243–249 (1939).

(7) DU VIGNEAUD, V.: The role which insulin has played in our concept of protein hormones, and a consideration of certain phases of the chemistry of insulin. *In* Cold Spring Harbor Symposia on Quantitative Biology, **6:** 275–285 (1938).

(8) WHITE, A.: Protein hormones. *In* Cold Spring Harbor Symposia on Quantitative Biology, **6:** 262–274 (1938).

(9) STERN, K. G.: The relationship between prosthetic group and protein carrier in certain enzymes and biological pigments. *In* Cold Spring Harbor Symposia on Quantitative Biology, **6:** 286–300 (1938).

(10) NORTHROP, J. H.: Crystalline enzymes: The chemistry of pepsin, trypsin, and bacteriophage. Columbia Biol. ser. No. 12. New York (1939).

(11) CLAUDE, A.: Chemical composition of tumor-producing fraction of chicken tumor I. Science, **90:** 213–214 (1939).

(12) LORING, H. S.: Properties and hydrolytic products of nucleic acid from tobacco mosaic virus. J. Biol. Chem., **130:** 251–258 (1939).

(13) STERN, K. G., and Ruran-Reynals, F.: Physico-chemical properties of Rous chicken tumor agent. Science, **89**: 609–610 (1939).

(14) WYCKOFF, R. W. G., and BEARD, J. W.: pH stability of Shope papilloma virus and of purified papilloma virus protein. Proc. Soc. Exper. Biol. & Med., **36:** 562–563 (1937).

(15) POLLARD, A.: Chemical composition of active agent of Rous sarcoma No. 1 and of some related products. Brit. J. Exper. Path., **20:** 429–438 (1939).

(16) MCFARLANE, A. S., MACFARLANE, M. G., AMIES, C. R., and EAGLES, G. H.: A physical and chemical examination of vaccinia virus. Brit. J. Exper. Path., **20:** 485–501 (1939).

(17) LUCK, J. M.: Liver proteins: question of protein storage. J. Biol. Chem., **115:** 491–510 (1936).

(18) ——— Liver proteins (the extractability of the liver proteins). Compt. rend. d. trav. du Lab. Carlsberg, **22** (Sorensar Jubilee vol.): 321–328 (1938).

(19) OSWALD, A.: Zur Chemie des Carcinonis. Ztschr. f. physiol. Chem., **97:** 264–268 (1916).

(20) STEUDEL, H.: Zusammengesetzte Einwirkungen, Proteide und ihre Bausteine. *In* Abderhalden's Handbuch der biologischen Arbeitsmethoden, Abt. 1, Teil 8, p. 1, Berlin (1922).

(21) MAVER, M. E.: Separation of cathepsin from malignant and normal rat tissue. J. Biol. Chem., **131:** 127–134 (1939).

(22) STEINHARDT, J. J.: The solubility of crystalline pepsin. I. Anomalous dependence of solubility on the solid to solvent ratio. J. Biol. Chem., **129:** 135–170 (1939).

(23) KELLEY, E. G.: Reactions of dyes with cell substances, quantitative comparison of tissue nuclei and extracted nucleoproteins. J. Biol. Chem., **127:** 55–71 (1939).

(24) HEIDELBERGER, M., and KENDALL, F. E.: Protein fractions of scarlatinal strain of Streptococcus hemolyticus. J. Exper. Med., **54:** 515–531 (1931).

(25) KOCH, F. C., and MCMEEKIN, T. L.: A new direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. J. Am. Chem. Soc., **46:** 2066–2069 (1924).

(26) KNOX, R.: Desiccation of filterable tumours and other biological materials. J. Path. & Bact., **49:** 467–481 (1939).

(27) LUGG, J. W. H.: On the use of mercuric salts and nitrous acid in the colorimetric determination of tyrosine and tryptophan present in solution. Biochem. J., **31:** 1422–1433 (1937).

(28) ——— Investigations of sources of error in the estimation of tyrosine and tryptophan in complex materials, which are associated with hydrolysis. Biochem. J., **32:** 775–783 (1938).

(29) BAERNSTEIN, H. D.: A new method for the determination of methionine in proteins. J. Biol. Chem., **115:** 25–32 (1936).

(30) BAILEY, K.: The sulphur distribution of proteins. Biochem. J., **31:** 1396–1405 (1937).

(31) GREENSTEIN, J. P.: Sulfhydryl groups in proteins. I. Egg albumin in solutions of urea, guanidine, and their derivatives. J. Biol. Chem., **125:** 501–513 (1938).

(32) ——— Sulfhydryl groups in proteins. II. Edestin, excelsin, and globin in solutions of guanidine hydrochloride, urea, and their derivatives. J. Biol. Chem., **128:** 233–240 (1939).

(33) ——— Sulfhydryl groups in proteins. III. The effect on egg albumin of various salts of guanidine. J. Biol. Chem., **130:** 519–526 (1939).

(34) STEINHARDT, J.: Properties of hemoglobin and pepsin in solutions of urea and other amides. J. Biol. Chem., **123:** 543–575 (1938).

(35) GREENSTEIN, J. P., with the technical assistance of JENRETTE, W. V.: Chemical studies on the components of normal and neoplastic tissues. I. Viscosity and streaming birefringence of sodium thymonucleate. J. Nat. Cancer Inst. 1: 83–96 (1940).

(36) HEIDELBERGER, M., and SCHERP, H. W.: Protein sections of a strain of group A hemolytic streptococci. III. Group A. J. Immunol., **37:** 563 (1939).

# LUNG TUMORS AND HEREDITY. I. THE SUSCEPTIBILITY OF FOUR INBRED STRAINS OF MICE AND THEIR HYBRIDS TO PULMONARY TUMORS INDUCED BY SUBCUTANEOUS INJECTION [1]

By W. E. HESTON, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

The inheritance of susceptibility to lung tumors in mice has not been completely explained, although much progress has been made. That susceptibility is dominant has been quite well established, and the possibility of its being controlled by only a single pair of genetic factors has been suggested.

As early as 1926, Lynch (*1*) suggested dominant inheritance of spontaneous lung tumors in mice, and later Bittner and Little (*2*) presented data showing that such tumors were inherited according to genetic principles and suggested that one or more dominant factors are probably involved. From still more recent data on the incidence of spontaneous lung tumors in strain A (susceptible), strain B (C57 black) (resistant) mice and their reciprocal $F_1$ and $F_2$ hybrids, Bittner (*3*) concluded that susceptiblity in mice to spontaneous pulmonary tumors is probably transmitted by a single dominant Mendelian factor.

In general, results of experiments on the inheritance of susceptibility to induced lung tumors have paralleled those for spontaneous tumors. Lynch (*4*) found that although tar painting increased the incidence of lung tumors, the relative susceptibility of the high and low strains of mice was not materially altered.

When Andervont (*5*) injected 1,2,5,6-dibenzanthracene [2] in lard solution subcutaneously into mice, he increased the lung-tumor incidence and greatly hastened the development of the tumors, yet the hereditary behavior appeared much the same as that of spontaneous tumors. He found that following the subcutaneous injection of the carcinogen, 90 percent of the first hybrid generation from reciprocal matings of A strain × B strain mice and 74.7 percent of the second hybrid generation developed lung tumors, which indicated that the character was inherited in a dominant manner. In later work (*6*) involving the backcross generation, 100 percent of the mice produced by mating the $F_1$ to the susceptible-strain parent and 45 percent of those produced by backcrossing the $F_1$ progeny to the resistant parent were found to be susceptible after injection. These results suggested that but one dominant factor may be involved in the inheritance of susceptibility to induced tumor when mice of strains A and B are used as test animals.

With such data available, it seemed desirable to determine how the character responded when the A strain was outcrossed to inbred resistant strains other than the C57 black strain. Such crosses would also be of value in that the $F_1$ progeny could be backcrossed to the resistant-parent strain which might reveal evidences of linkage between the gene or genes for lung tumor and any carried by the resistant strains. This paper presents data obtained by outcrossing the A strain to the L, N, and W strains.

[1] *This work was supported by the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and was conducted at that laboratory.*

[2] *Hereinafter referred to as dibenzanthracene.*

## STOCKS

Following is a brief description of each of the four stocks employed:

*A strain:* This stock was obtained from Dr J. J. Bittner. It is the same stock as that used so extensively by Bittner and Andervont in their lung-tumor studies. The animals have been inbred by brother-sister matings over 55 generations, and the incidence of spontaneous lung tumors as recorded by Bittner (*3*) is 89.2 percent for virgin females at an average age of 19.5 months, and 74.4 percent for males at an average age of 16.1 months. They carry albinism, brown, nonagouti, and the normal alleles of the genes for leaden, piebald, and dilution.

*L (leaden) strain:* This strain was obtained from Dr. A. M. Cloudman who states that he has observed no lung tumors in the strain since he secured the stock from Dr. J. M. Murray in 1934. The leaden mutation *ln*, described by Murray (1935), arose in the C57 brown stock which in turn was derived from the progenitors of the C57 black stock. Brother-sister inbred generations total over 40 since the original female (No. 57), and over 20 since the leaden mutation arose. Their genotype is *CCaabblnln*.

*N strain:* This strain also was secured from Dr. J. J. Bittner, who describes the strain as being resistant to spontaneous lung tumors. Strong originally developed the strain, but it has since been outcrossed to the C57 black stock. Ten generations of inbreeding by brother-sister matings have occurred since the outcross. The strain carries the dilution *d* and the piebald *s* genes. Thus, their genotype is *CCaabbddss*.

*W strain:* This stock was likewise obtained from Dr. Bittner, who found it to be low in spontaneous lung-tumor susceptibility. He discovered the light-bellied mutation, which characterizes the strain, in a hybrid from the mating $C_3H \times N$. The stock carries the full color and black genes. It has been inbred over 20 generations by brother-sister matings.

## EXPERIMENTAL PROCEDURE

In view of the fact that former work has shown that induced lung tumors tend to behave genetically as do spontaneous ones, yet appear as much as 18 months earlier, it seemed advisable to work with induced lung tumors since results could be obtained much more rapidly. The carcinogenic agent employed, dibenzanthracene, was dissolved in olive oil at 110° C., at a concentration of 4 mg. of dibenzanthracene in 1 cc. of olive oil. Each mouse received on the back a subcutaneous injection of one-fourth cubic centimeter of the solution (1 mg. of the carcinogen) cooled to approximately 40° C. After the injection needle was removed, the globule was palpated to facilitate absorption of the carcinogen.

All mice were injected at the age of 2 months (56 days) and with the exception of a group of strain A and two groups of backcross animals, all were autopsied at 168 days of age, or 4 months (112 days) after the injection. The time for autopsying was set at 168 days since it was found that if the mice were permitted to live longer, large subcutaneous tumors arose at the site of the injection, especially in the W stock, necessitating the autopsy of the animals. Thus, the age at autopsy would not have been uniform, and complications would have been added to the analysis of the data.

Diagnosis of the presence of lung nodules was done with the aid of the dissecting microscope, and an approximate count was made of the number of nodules appearing on the surface of the lungs.

The lung-tumor incidence was estab-

lished for each of the four inbred strains. $F_1$ hybrids were reared by crossing strain A to each of the three resistant strains, and these hybrids were in turn tested. $ALF_1$[3] females were backcrossed to L males to produce an AL ♀–LBC generation which was used to test for tumor incidence and also for linkage between lung-tumor susceptibility and the leaden gene *ln*. Likewise, an AN ♀–NBC generation was reared for studies of linkage between lung-tumor susceptibility and the piebald gene *s*, or the dilution gene *d*. In the third case, it was desirable to cross the $AWF_1$ mice with a resistant strain homozygous for nonagouti in order to establish whether or not lung-tumor susceptibility was linked with the agouti gene locus. The N stock fitted the requirement and was therefore employed.

Since there was the possibility that breeding might introduce complicating factors, most prominent of which would have been the development of mammary-gland tumors, all females used for testing tumor incidence were kept as virgins and none of the test males were used for breeding.

As it had been established that there was no maternal influence affecting lung-tumor transmission and that the factor or factors were not sex-linked, it seemed unnecessary to make reciprocal matings or to separate the sexes in calculating the tumor incidence. However, in the groups of test mice the sexes were approximately equal in number.

[3] *The following symbols are used here and elsewhere in this paper:*

*$ALF_1$—hybrid produced by mating A ♀ × L ♂.*

*$ANF_1$—hybrid produced by mating A ♀ × N ♂.*

*$AWF_1$—hybrid produced by mating A ♀ × W ♂.*

*$WAF_1$—hybrid produced by mating W ♀ × A ♂.*

*AL ♀–LBC—hybrid produced by backcrossing $ALF_1$ ♀ × L ♂.*

*AN ♀–NBC—hybrid produced by backcrossing $ANF_1$ ♀ × N ♂.*

*AW ♀–N—hybrid produced by outcrossing $AWF_1$ ♀ × N ♂.*

## TUMOR INCIDENCE

The lung-tumor incidence, following the technique described above, of each of the four inbred strains and their hybrids is presented in table 1. At 112 days after the subcutaneous injection of 1 mg. of dibenzanthracene, 100 percent of the A strain mice had developed lung nodules, while the leaden strain showed 100-percent resistance. The N and W strains were also resistant, although neither showed total resistance. The N strain yielded tumors in 7.22 percent of the animals, and the W strain in 32.98 percent.

TABLE 1.—*Incidence of lung tumors in mice injected with 1 mg. of dibenzanthracene at 56 days of age and autopsied at 168 days of age*

| Type of mice | Number | Mice that developed lung tumors | | Mice that did not develop lung tumors | |
|---|---|---|---|---|---|
| | | *Number* | *Percent* | *Number* | *Percent* |
| A | 113 | 113 | 100.00 | 0 | 0.00 |
| L (leaden) | 97 | 0 | 0.00 | 97 | 100.00 |
| $ALF_1$ | 114 | 107 | 93.86 | 7 | 6.14 |
| AL ♀–LBC | 125 | 22 | 17.60 | 103 | 82.40 |
| N | 97 | 7 | 7.22 | 90 | 92.78 |
| $ANF_1$ | 102 | 76 | 74.51 | 26 | 25.49 |
| AN ♀–NBC | 113 | 35 | 30.97 | 78 | 69.03 |
| W | 94 | 31 | 32.98 | 63 | 67.02 |
| $AWF_1$ and $WAF_1$ | 131 | 131 | 100.00 | 0 | .00 |
| AW ♀–N | 137 | 69 | 50.36 | 68 | 49.64 |

Matings of females of the 100-percent-susceptible strain (A) to males of the 100-percent-resistant strain (L) produced $F_1$ progeny which were 93.86 percent susceptible, and the $F_1$ progeny of A ♀ × N ♂ were 74.51 percent susceptible. Both percentages were high, but neither reached the degree of susceptibility displayed by the A stock. However, $F_1$ hybrids produced by mating A strain to W strain animals developed lung tumors in 100 percent of the 131 animals tested. Yet it should be noted that in this cross the strain considered as resistant yielded as much as 32.98-percent susceptibility.

The 125 mice produced by backcrossing

the $F_1$ progeny of A♀×L♂ to the resistant-parent strain (L) yielded a degree of susceptibility as low as 17.6 percent when subjected to the same procedure as that which had effected total susceptibility in the one $P_1$ stock and total resistance in the other. Likewise, AN♀–NBC mice, produced by mating $ANF_1$ females to N males were only 30.97 percent susceptible. When Andervont backcrossed mice of the $F_1$ generation with C57 black, he obtained a generation which showed 45-percent susceptibility which he reports as suggesting single-dominant-factor inheritance, but his mice were permitted to run longer before being autopsied. The AL♀–LBC and AN♀–NBC mice developed a higher incidence of lung tumors when the carcinogen was given a longer time in which to act. When autopsied 6 months (168 days) after the injection, 31 AL♀–LBC mice gave a tumor incidence of 29.03 percent, and 34 AN♀–NBC mice an incidence of 73.56 percent. These figures tend to indicate that the ability to get a 50:50 ratio, which would be expected in such a backcross when dealing with a single-factor dominant, is dependent upon the duration of time following the injection of the carcinogen.

## LINKAGE

The backcross generations have been classified according to their genotypes in order that any evidence for or against linkage might be observed (table 2). It is quite evident that lung tumor is not linked with any of the five genes tested. If there had been linkage with the *ln* (leaden) locus, one would have expected that lung-tumor susceptibility would come in with the *Ln* gene from the strain A mice, thus making a higher percentage among the *Lnln* mice than among the *lnln* animals. Since the reverse of this was true, there is evidence of no linkage here. The lung-tumor percentages were almost identical in the AN♀–NBC groups, thus failing to give evidence of linkage with either the *d* or the *s* gene. There is slight indication of linkage to the nonagouti gene, but this is not supported by the nontumor incidence in the same group. In considering the gene for brown, it is noted that *Bb* mice show a higher tumor incidence than do *bb* mice, the reverse of that expected if linkage occurred. No two of these genes are carried on the same chromosome. Genes on other chromosomes are now being tested.

TABLE 2.—*Linkage tests in mice of various backcrosses*

| Crosses | Progeny | Genotype | Mice with lung tumors | | Mice without lung tumors | |
|---|---|---|---|---|---|---|
| | *Number* | | *Number* | *Percent* | *Number* | *Percent* |
| $ALF_1$♀×L♂ | 125 | *Lnln* | 9 | 7.20 | 46 | 36.80 |
| | | *lnln* | 13 | 10.40 | 57 | 45.60 |
| | | *Dd* | 18 | 15.93 | 32 | 28.32 |
| $ANF_1$♀×N♂ | 113 | *dd* | 17 | 15.04 | 46 | 40.71 |
| | | *Ss* | 18 | 15.93 | 46 | 40.71 |
| | | *ss* | 17 | 15.04 | 32 | 28.32 |
| | | *A=a* | 29 | 21.17 | 32 | 23.35 |
| $AWF_1$♀×N♂ | 137 | *aa* | 40 | 29.20 | 36 | 26.28 |
| | | *Bb* | 37 | 27.01 | 31 | 22.63 |
| | | *bb* | 32 | 23.35 | 37 | 27.01 |

## DEGREE OF SUSCEPTIBILITY

The degree of susceptibility may be revealed by counting the number of nodules appearing on the surface of the lungs of each animal at autopsy (*7*, *8*, *9*). In this experiment, each mouse was classified in one of five groups depending upon the number of nodules it possessed, and from the percentages of animals falling in each group the histograms (fig. 1) were constructed. It will be noted that with each outcross the hybrids which developed lung tumors did not have as many nodules as did the animals of the A strain, but instead they fell as intermediates between the degree represented by the A strain and that represented by the resistant strains. Furthermore, the tumor-bearing mice of the groups produced by backcrossing the

$F_1$ progeny to the resistant parents fell as intermediates between their parent groups.

Susceptibility might also be measured by the time required for the nodules to develop (*10*). What we think of as a susceptible strain might be defined as one in which the nodules appear early, and a resistant strain as one requiring a longer time for the nodules to develop, not all resistant strains possessing the same degree of resistance.

At 56 days (2 months) after the injection, 61 strain A mice were autopsied, and all had developed nodules; at 28 days (1 month) after the injection, 23 strain A mice were autopsied, and all were negative with the exception of 2 animals, each of which showed 1 small nodule. Thus, the degree of susceptibility of the strain A mice might be defined as falling between the twenty-eighth and the fifty-sixth day after the injection. Andervont (*10*) found lung tumors beginning to appear at 4 weeks following injection in strain A mice, and by the eighth week virtually all animals had nodules. Grady and Stewart (*11*) in studying the histogenesis of induced pulmonary tumors in strain A mice found the tumors beginning to appear 5 weeks after the injection of 0.8 mg. of dibenzanthracene in lard solution.

Early in the experiment 21 mice of the W strain were autopsied at 140 days (5 months) after they had been injected, and 17, or 80.95 percent, of them displayed lung tumors, whereas only 32.98 percent of the same strain had developed nodules 4 months after the injection. This gives some indication of the degree of susceptibility of the W strain although the limits are not so clearly defined as in the case of the A strain. With this technique it would be impossible to define these limits clearly because of the appearance of the subcutaneous tumors, which cause some of the mice to expire early after the injection.

The 31 AL ♀ –LBC and 34 AN ♀ –NBC mice mentioned above, which were kept until 6 months after the injection before they were autopsied, showed a tumor incidence of 29.03 percent for the former

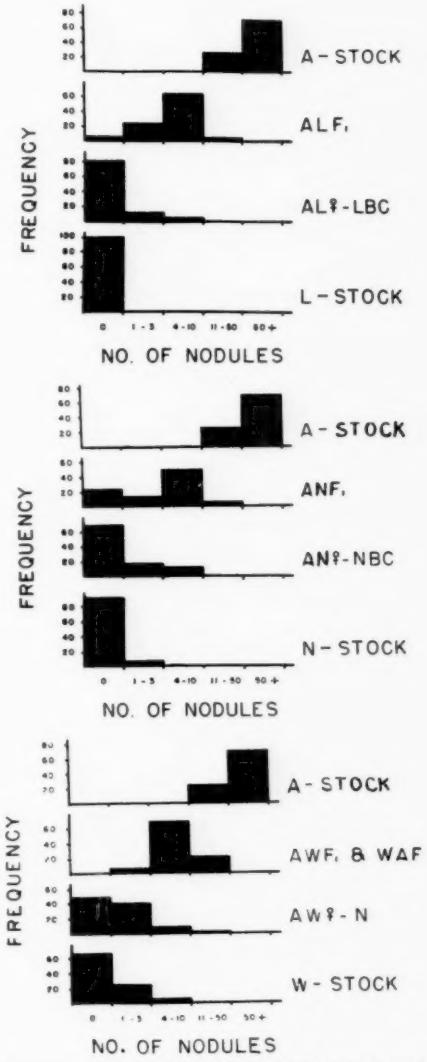



FIGURE 1.—Mice of the four inbred strains and their hybrids, grouped according to the number of lung nodules per individual.

group and 73.56 percent for the latter. Thus, it is evident that some of these backcross animals did not develop nodules until after they had been injected 4 months or longer, because at 4 months they had shown incidences of 17.6 and 30.97 percent, respectively (table 1).

## DISCUSSION

The results fail to support a theory of one-factor Mendelian inheritance of susceptibility to induced lung tumor. Although the incidence of tumor among the $ALF_1$ animals did not deviate far from the 100-percent susceptibility expected with a single dominant factor, the incidences in the $ANF_1$ and especially in the backcross groups were too low to uphold such a theory. The experiments tend more to suggest the action of multiple factors.

The data gathered by counting the number of lung nodules presented results that could have been expected if dealing with multiple factors. The $F_1$ fell as intermediate between the A and the resistant strains, and the backcross to the resistant as intermediate between the $F_1$ and the resistant. Such data certainly merit further testing with the $F_2$ and the backcross to the susceptible strain employing identical procedure. Lynch (*9*) has recently published data which show that the degree of susceptibility to lung tumors, as measured by the number of nodules occurring in the individual, is affected by heredity, but the number of genes concerned is not revealed.

Factors influencing time of development of the nodules were apparent. Strain A mice developed nodules between 1 and 2 months after the injection, while some of the W strain did not develop tumors until after 4 months. Also, the nodules in some of the backcross animals did not appear until between the fourth and sixth month following the injection.

That there were nongenetic factors affecting the development of the tumors was evidenced by the fact that 32.98 percent of the W strain, inbred for 20 generations, showed lung tumors, while 67.02 percent of the same strain were negative (table 1) although the same procedure had been applied to all animals. In general the same was true of the N strain.

It is not inconceivable that a theory of multiple-factor inheritance could follow the $F_1$, $F_2$, and backcross data obtained by Bittner and Andervont suggesting single dominant inheritance. While studying crosses between two inbred strains of guinea pigs, differing in number of digits, Wright (*12*) obtained $F_1$, $F_2$, and backcross ratios which might have been interpreted as inheritance due to a single recessive factor. However, Wright showed that such an interpretation broke down completely when tests of the supposed segregants were made. The explanation which followed was that the strains differed in several factors and that the character approached alternative expression because of physiological thresholds. In the case of lung-tumor inheritance several factors might be revealed by breeding tests of the $F_2$ or backcross segregants.

The results of these experiments suggest that one should consider susceptibility to induced lung tumors as manifested in a degree which may vary from the condition found in the most resistant strains to that of the most susceptible strains. Andervont (*10*) has made similar observations for he states: "The difference of susceptibility to induced pulmonary tumors, as exhibited by the various strains of mice, is a matter of degree and it is suggested

that hereditary factors exert their influence by controlling the degree of susceptibility." It is with this in mind that future analysis of the inheritance of induced lung tumors in mice should be undertaken.

## SUMMARY

The data obtained by outcrossing the strain A mice to those of the resistant strains L, N, and W failed to give evidence for single Mendelian inheritance of susceptibility to induced lung tumors, but did suggest the action of multiple factors.

It was pointed out that nongenetic factors may influence the development of induced lung tumors.

There was no evidence of linkage between lung-tumor susceptibility and the gene for leaden, piebald, dilution, agouti, or brown.

NOTE.—The author is indebted to Dr. J. J. Bittner for general consultation and for suggesting certain phases of the experiments.

## REFERENCES

(*1*) LYNCH, C. J.: Studies on the relation between tumor susceptibility and heredity. III. Spontaneous tumors of the lung in mice. J. Exper. Med., **43**: 339–355 (1926).

(*2*) BITTNER, J. J., and LITTLE, C. C.: The transmission of breast and lung cancer in mice. J. Hered., **28**: 117–121 (1937).

(*3*) BITTNER, J. J.: Spontaneous lung carcinoma in mice. Pub. Health Rep., **53**: 2197–2202 (1938).

(*4*) LYNCH, C. J.: Studies on the relation between tumor susceptibility and heredity. IV. The inheritance of susceptibility to tar-induced tumors in the lungs of mice. J. Exper. Med., **46**: 917–933 (1927).

(*5*) ANDERVONT, H. B.: Pulmonary tumors in mice. II. The influence of heredity upon lung tumors induced by the subcutaneous injection of a lard-dibenzanthracene solution. Pub. Health Rep., **52**: 304–315 (1937).

(*6*) ——— Pulmonary tumors in mice. V. Further studies on the influence of heredity upon spontaneous and induced lung tumors. Pub. Health Rep., **53**: 232–237 (1938).

(*7*) SHIMKIN, M. B.: Induced pulmonary tumors in mice. I. Susceptibility of seven strains of mice to the action of intravenously injected methylcholanthrene. Arch. Path., **29**: 229–238 (1940).

(*8*) ——— Induced pulmonary tumors in mice. II. Reaction of lungs of strain A mice to carcinogenic hydrocarbons. Arch. Path., **29**: 239–255 (1940).

(*9*) LYNCH, C. J.: Influence of heredity and environment upon number of tumor nodules occurring in lungs of mice. Proc. Soc. Exper. Biol. & Med., **43**: 186–189 (1940).

(*10*) ANDERVONT, H. B.: Pulmonary tumors in mice. VI. Time of appearance of tumors induced in strain A mice following injection of 1,2,5,6-dibenzanthracene or 20-methylcholanthrene. Pub. Health Rep., **54**: 1512–1519 (1939).

(*11*) GRADY, H. G., and STEWART, H. L.: Origin of induced pulmonary tumors in strain A mice. (Abstract) Pub. Health Rep., **55**: 169–170 (1940).

(*12*) WRIGHT, S.: The results of crosses between inbred strains of guinea pigs, differing in number of digits. Genetics, **19**: 537 (1934).

# GLUTAMIC ACID FROM NORMAL AND CANCEROUS TISSUE

By J. M. JOHNSON, *senior chemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

The question of a difference between the amino acids present in cancer tissue and those present in normal tissue was raised last year by Kögl and Erxleben (*1*). These investigators advanced the claim that certain amino acids isolated from the hydrochloric acid hydrolysates of malignant tissue (ovarian tumor, mammary carcinoma, etc.) differed in the values for their optical rotations from the same amino acids isolated from normal tissue (such as heart, lung, or ovary). Certain amino acids from cancerous tissues were found to be partly racemized, that is, instead of having the optical rotation of the ordinary or natural form, they showed a lesser rotation which indicated the presence of the unnatural form to a certain degree. This difference was most marked in the case of glutamic acid. The ordinary or natural form of glutamic acid has a specific rotation of $[AC]_D = +31.5°$ and is referred to as *l* (+)-glutamic acid; Kögl and Erxleben found glutamic acid isolated from cancer-tissue hydrolysates which possessed rotations of +21.8°, +4.6°, +11.6°, and +16.1°. From these figures they calculated the amounts of *d* (−)-glutamic acid present to be from 15.6 to 42.7 percent, whereas normal tissue yielded glutamic acid with the rotation very close to +31.5°. From this they concluded that the presence of *d* (−)-glutamic acid in tissue hydrolysates is associated with malignancy.

Since the appearance of this paper several other workers have reported the results of their work on this glutamic acid problem (*2–14*). Some agree and some disagree with Kögl and Erxleben. In subsequent papers Kögl and coworkers (*15–18*) have reaffirmed their first view on the subject.

## MATERIALS AND METHODS

It seemed desirable to extend this investigation to a tumor hitherto not studied, the Jensen rat sarcoma. Furthermore, the normal livers of animals bearing this tumor as well as the livers from normal animals were used for comparison. Fifteen Jensen sarcoma tumor (intramuscular) rats were killed and immediately perfused through the descending aorta with modified Ringer's solution. The tumor tissue was cut out, and all necrotic and muscle tissue was discarded. The livers, which were now free of blood, were also dissected out. A number of normal rats were also killed and their livers obtained in the same manner. The tissues were ground with alcohol, filtered off, washed with alcohol and ether, and dried in a vacuum desiccator over sulfuric acid. The dried material thus obtained was hydrolyzed by heating to gentle boiling under reflux with four volumes of 20-percent hydrochloric acid for 30 hours. The cooled hydrolysates were filtered through asbestos and concentrated under 45° C. and reduced pressure to a thick sirup, cooled with a salt-ice mixture and saturated with dry hydrochloric acid gas. The solution was kept in a stoppered flask in a subzero chest for several days, or until crystallization no longer increased. The crystals were filtered off with the aid of a sintered glass crucible in the cold room,

washed with ice cold saturated hydrochloric acid solution, and dried in a vacuum desiccator over soda lime to constant weight. The mother liquors and washings were concentrated under reduced pressure at 45° C. or below, to almost complete dryness. Water was added and the solution again concentrated as before to practical dryness. The residue was dissolved in water again and a large part of the color removed with phosphotungstic acid. The excess phosphotungstic acid was removed from the filtrate with amyl alcohol and ether mixture. The aqueous solution remaining was concentrated under reduced pressure at low temperature and treated with barium hydroxide at room temperature until neutral to litmus. The barium salt so formed was precipitated in the cold with five volumes of 95-percent alcohol. Barium was exactly removed from the precipitate with sulfuric acid, the filtrate from the barium sulfate was concentrated under reduced pressure below 45° C. to a thick sirup. The latter was cooled with a salt-ice mixture and saturated with dry hydrochloric acid gas. The crystals thus obtained were combined with those obtained directly by hydrochloric acid gas saturation of the hydrolysate. This additional procedure applied to the mother liquors is the Jones and Moeller modification (*19*) of the Foreman (*20*) technique. The combined crystals are those given in table 1 as "crude glutamic acid hydrochloride."

The crude glutamic acid hydrochloride was recrystallized from 20-percent hydrochloric acid to constant specific rotation and in each case the major portion of the crystals proved to be *l* (+)-glutamic acid. The mother liquors from the recrystallization of the crude glutamic acid hydrochloride upon concentration under reduced pressure below 45° C., yielded further crops of glutamic acid hydrochloride on cooling and saturation with hydrochloric acid gas. These later crops, whether isolated from normal tissue hydrolysates or from tumor tissue hydrolysates invariably revealed low rotations. This indicated the presence of small amounts of *d* (−)-glutamic acid in the hydrolysates from all tissues. The presence of cystine hydrochloride as a cause of the low rotations was ruled out each time by a nitroprusside test after reduction by sodium cyanide.

Livers of normal rabbits were treated in the same way as in the case of normal

TABLE 1.—*Glutamic acid analyses of tumor and liver of Jensen sarcoma rats and the liver of normal rats*

| Material | Jensen sarcoma rats | | | | | | Normal rats—Liver | | |
|---|---|---|---|---|---|---|---|---|---|
| | Tumor | | | Liver | | | | | |
| | Weight | $[\alpha]D^{20}$ in 9-percent HCl | *d* form | Weight | $[\alpha]D^{20}$ in 9-percent HCl | *d* form | Weight | $[\alpha]D^{20}$ in 9-percent HCl | *d* form |
| | *Grams* | *Degrees* | *Percent* | *Grams* | *Degrees* | *Percent* | *Grams* | *Degrees* | *Percent* |
| Fresh | 149 | | | 154 | | | 211 | | |
| Dried | 20 | | | 43 | | | 45 | | |
| Crude glutamic acid hydrochloride | 2.6 | | | 2.1 | | | 5.47 | | |
| Recrystallized three times | .774 | +31.4 | | .929 | +31.7 | | 2.40 | +31.5 | |
| Concentrated mother liquors of above | 1.376 | +12.8 | | | | | .858 | +29.8 | |
| Recrystallized once | .478 | +15.6 | | .588 | +23.9 | | .649 | +31.1 | |
| Recrystallized twice | .251 | +17.2 | 23.0 | .384 | +27.1 | 7.1 | | | |
| Crystals from mother liquors of above | .262 | +14.9 | 26.3 | .360 | +17.0 | 23.0 | .538 | +2.4 | |
| Recrystallized again | | | | | | | .310 | +1.88 | 47.0 |
| Calculated for total crude glutamic acid | | | 2.6 | | | 3.9 | | | 2.4 |
| *d*-form in the dried weight | | | .61 | | | .25 | | | .32 |
| Pure (+)-glutamic acid hydrochloride | | +31.5 | | | | | | | |

rats, except that hydrolysis was effected by heating for 30 hours with three volumes of concentrated hydrochloric acid (sp. gr. 1.19). The crude glutamic acid hydrochloride was recrystallized and several crops of crystals were obtained from the mother liquors, using the same methods previously described. The results are given in table 2.

TABLE 2.—*Glutamic acid analyses of the liver of normal rabbits*

| Material | Weight | $[\alpha]_D$ in 9-percent HCl | *d* form | *d* form calculated |
|---|---|---|---|---|
| | *Grams* | *Degrees* | *Percent* | *Gram* |
| Fresh | 967 | | | |
| Dried | 237 | | | |
| Crude glutamic acid hydrochloride | 25.957 | | | |
| Crystals: | | | | |
| First crop | 11.3234 | +31.7 | | |
| Second crop | 1.3889 | +.95 | 48.5 | 0.674 |
| Third crop | .2436 | +7.44 | 38.2 | .093 |
| Fourth crop | .1283 | +7.1 | 38.7 | .050 |
| Fifth crop | .5457 | +8.6 | 36.3 | .198 |
| Sixth crop | .1128 | +1.2 | 48.1 | .054 |
| Seventh crop | .2027 | +3.5 | 44.4 | .090 |
| Calculated for total crude glutamic hydrochloride | | | 4.5 | |
| *d* form in the dried weight | | | .49 | |

These results indicate that the greater proportion of glutamic acid isolated from hydrochloric acid hydrolysates, both of normal and tumor tissue is in the natural *l* (+)-form. The *d* (−)-form occurs only in from 2.4 to 4.5 percent of the total crude glutamic acid hydrochloride isolated. Or, as Chibnall and coworkers (*2*) have pointed out, it is best to make the calculation on the basis of percent of dried tissue material. Our results show that percentages of *d* (−)-form were 0.25, 0.32, 0.62, and 0.49 when the total glutamic acid hydrochloride isolated ranged from 5 to 13 percent of the dried tissue. Therefore the *d* (−)-form is either present in this small proportion in all tissues, normal as well as cancerous, or it has been formed by racemization of the *l* (+)-form during the process of hydrolysis of the protein.

In order to decide this latter point pure *l* (+)-glutamic acid hydrochloride was subjected to prolonged gentle boiling (35.5 hours) with three volumes of concentrated hydrochloric acid (sp. gr. 1.19). These are the proportions and concentration of acid used by Kögl and Erxleben in their hydrolysis of tissues, although they heated only 7 hours. At the end of the heating, the liquid was cooled in the cold room (4° C.) for several days, the crystals which had deposited were filtered off on a sintered glass crucible, washed with ice cold saturated hydrochloric acid solution, and dried to constant weight in a vacuum desiccator over soda lime and sulfuric acid. This represented the first crop of crystals. Succeeding crops were obtained by concentration of the mother liquors under reduced pressure at a temperature below 45° C., and by saturation of the concentrate at 0° C. with hydrochloric acid gas. There was 99.67-percent recovery of the glutamic acid hydrochloride used, of which 12.934 gm., or 4.6 percent, had been converted into the *d* (−)-glutamic acid or the so-called unnatural form. In table 3 are recorded the results.

TABLE 3.—*Recovery of glutamic acid hydrochloride after being heated with three volumes of hydrochloric acid 35.5 hours*

| Crop | Weight | $[\alpha]_D$ in 9-percent HCl | Nitrogen | *d*-form | *d*-form calculated | Recovery |
|---|---|---|---|---|---|---|
| | *Grams* | *Degrees* | *Percent* | *Percent* | *Grams* | *Percent* |
| Original | 280 | +31.67 | 7.58 | | | |
| First | 256 | +31.17 | 7.55 | | | 91.43 |
| Second | 21.9 | −4.26 | 7.55 | 56.7 | 12.42 | 7.82 |
| Third | .90 | +2.23 | 7.49 | 46.4 | .42 | .32 |
| Fourth | .274 | +9.9 | 7.56 | 34.3 | .094 | .10 |

Another experiment was carried out whereby the glutamic acid hydrochloride was heated for only 7 hours to gentle boiling with three volumes of concentrated hydrochloric acid (sp. gr. 1.19) and the resulting solution crystallized in suc-

cessive crops as before. Of the glutamic acid hydrochloride used, 98.76 percent was recovered, of which 4.956 gm., or 1.9 percent, had been converted into the *d*-form. Table 4 gives the results.

TABLE 4.—*Recovery of glutamic acid hydrochloride after being heated with three volumes of hydrochloric acid 7 hours*

| Crop | Weight | $[\alpha]_D$ in 9-percent HCl | Nitrogen | *d*-form | *d*-form calculated | Recovery |
|---|---|---|---|---|---|---|
| | *Grams* | *Degrees* | *Percent* | *Percent* | *Grams* | *Percent* |
| Original | 256 | +31.17 | 7.55 | | | |
| First | 238 | +31.26 | 7.58 | | | 92.97 |
| Second | 14.1 | +10.57 | 7.55 | 33.2 | 4.68 | 5.51 |
| Third | .5395 | +9.9 | 7.59 | 34.3 | .185 | .21 |
| Fourth | .1915 | +1.6 | 7.62 | 47.4 | .091 | .07 |

It can be seen therefore that *l*(+)-glutamic acid may be racemized slightly by gentle heating with hydrochloric acid. This may be the explanation of the finding of *d*(−)-glutamic acid in tumor tissue hydrolysates as well as in normal tissue hydrolysates under the conditions of hydrolysis as used by Kögl and Erxleben. There appears to be no qualitative difference between the glutamic acid found in the hydrolysates of cancerous tissue and normal tissue.

## SUMMARY

The principal amount of glutamic acid found in tumor-tissue hydrolysate as well as in normal-tissue hydrolysate was the natural *l* (+)-form.

Small amounts of racemic glutamic acid mixtures were found in the hydrolysates of both normal tissue and tumor tissue.

Pure *l* (+)-glutamic acid was found to be slightly racemized after heating with hydrochloric acid. This may account for the presence of *d* (−)-glutamic acid found in acid hydrolysates of normal and cancerous tissues.

## REFERENCES

(*1*) KÖGL, F., and ERXLEBEN, H.: Zur Ätiologie der malignen Tumoren. 1. Mitteilung über die Chemie der Tumoren. Ztschr. f. physiol. Chem., **258**: 57–95 (1939).

(*2*) CHIBNALL, A. C., REES, M. W., TRISTAM, G. R., WILLIAMS, E. F., and BOYLAND, E.: The glutamic acid of tumour proteins. Nature, London, **144**: 71–72 (1939).

(*3*) ARNOW, L. E., and OPSAHL, J. C.: The configuration of the glutamic acid of adenocarcinoma protein. Science, **90**: 257–258 (1939).

(*4*) Graff, S.: Glutamic acid in malignant tumors. J. Biol. Chem., **130**: 13–17 (1939).

(*5*) CHARGAFF, E.: The configuration of glutamic and aspartic acids from pathogenic bacteria (*Phytomonas tumefaciens* and *Corynebacterium diphtheriae*). J. Biol. Chem., **130**: 29–33 (1939).

(*6*) LIPMANN, F., BEHRENS, O. K., KABIT, E. A., and BURK, D.: The determination of the total d-amino acid content of human tumors and normal tissues by means of d-amino acid oxidase. Science, **91**: 21–23 (1940).

(*7*) CHIBNALL, A. C., REES, M. W., WILLIAMS, E. F., and BOYLAND, E.: The glutamic acid of normal and malignant tissue proteins. Biochem. J., **34**: 285 (1940).

(*8*) OTTAWA, H.: Zur Frage der optischem Aktivität der aus Tumoreiweiss isolierten Glutaminsaüre. Ztschr. f. Krebsforsch., **49**: 677 (1940).

(*9*) WHITE, J., and WHITE, F. R.: On the nature of the glutamic acid of malignant tumor tissue. (Letter to editor). J. Biol. Chem., **130**: 435 (1939).

(*10*) CHIBNALL, A. C., REES, M. W., WILLIAMS, E. F., and BOYLAND, E.: Glutamic acid of proteins. Nature, London, **145**: 311–312 (1940).

(*11*) KONIKOVA, A.: Glutamic acid of proteins. Nature, London, **145**: 312 (1940).

(*12*) TOWN, B. W.: Glutamic acid of proteins. Nature, London, **145**: 312–313 (1940).

(*13*) DITTMAR, C.: The presence of *d* (−)-glutamic acid in tumor proteins. Ztschr. f. Krebsforsch., **49**: 397, 441 (1939). [Abstract in Chem. Abs., **34**: 1731 (1940)].

(*14*) JOHNSON, J. M.: A comparison of the optical forms of glutamic acid from normal and cancerous tissue. J. Biol. Chem., **132**: 781 (1940).

(*15*) KÖGL, F., and ERXLEBEN, H.: The glutamic acid of tumour proteins. Nature, London, **144**: 111 (1939).

(*16*) KÖGL, F., ERXLEBEN, H., and AKKERMAN, A. M.: Isolierung der Glutaminsäure aus Tumorproteinen. 2. Mitteilung über die Chemie der Tumoren. Ztschr. f. physiol. Chem., **261:** 141–153 (1939).

(*17*) KÖGL, F., and ERXLEBEN, H.: Zur Stereochemie der Proteine von myomen und anderen Geschwulsten. 3. Mitteilüng über die chemie der Tumoren. Ztschr. f. phvsiol. Chem., **261:** 154–171 (1939).

(*18*) KÖGL, F., ERXLEBEN, H., and HERKEN, H.: The chemistry of tumors. 4. Further stereochemical analyses of tumor proteins. Ztschr. f. physiol. Chem., **263:** 107 (1940). [Abstract in Chem. Abs., **34:** 3353 (1940)].

(*19*) JONES, D. B., and MOELLER, O.: Some recent determinations of aspartic and glutamic acids in various proteins. J. Biol. Chem., **79:** 429–441 (1928).

(*20*) FOREMAN, F. W.: Quantitative estimation of aspartic and glutamic acids in the products of protein hydrolysis. Biochem. J., **8:** 463–480 (1914).

249431—40——9

# CARCINOGENIC POTENCY OF STILBESTROL AND ESTRONE IN STRAIN $C_3H$ MICE

By MICHAEL B. SHIMKIN, *assistant surgeon*, and HUGH G. GRADY, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

The estrogenic property of stilbestrol (4:4′ dihydroxy-$\alpha$:$\beta$ diethylstilbene) was established in 1938 by Dodds and his coworkers (*1*). The numerous papers on the compound uniformly confirm its estrogenic activity, and affirm that it is similar to the natural estrogens in all its diverse effects. Stilbestrol, however, is relatively much more potent by the oral route of administration than are the natural estrogens. This property and its low cost have stimulated its clinical use (*2*).

The development of mammary carcinoma in mice treated with massive doses of natural estrogens was reviewed by Gardner (*3*). Two investigators recorded that stilbestrol has the property of inducing breast tumors in mice and rats. Lacassagne (*4*) obtained breast cancer in two male mice of the R3 strain in 84 and 112 days, respectively, after the biweekly administration of 25 gamma of stilbestrol subcutaneously, and noted that one of the tumors appeared much sooner than it would have after the injections of the same doses of estrone. Geschickter (*5*) produced mammary cancer in a rat injected subcutaneously with 200 gamma of stilbestrol daily for 100 days.

The following investigation, initiated in November 1938, was undertaken to compare the carcinogenic potency of stilbestrol and estrone (3-hydroxy 17-keto $\Delta$ 1, 3, 5-estratriene), and to add data concerning the pathologic effects in mice of the long-continued administration of large doses of the new estrogenic substance.

## EXPERIMENTAL PROCEDURE

The mice of strain $C_3H$ used in the experiment were bred and raised in this laboratory by Dr. H. B. Andervont. Practically all of the virgin females of this line develop spontaneous breast tumors at an average age of 11 months (*6*). Spontaneous hepatoma and primary pulmonary tumors are observed in 40 and 8 percent, respectively, in male mice over 15 months of age (*7*).

The animals were divided according to sex and were maintained under identical environmental conditions. The diet consisted of Purina dog-chow pellets and an unlimited supply of water. The mice were 5 to 6 weeks old at the start of the experimental procedures.

Thirty $C_3H$ virgin females, obtained from 8 litters, were divided into 3 groups so that litter mates were present in each group. Ten mice were injected subcutaneously with 0.05 mg. of estrone in 0.25 cc. of purified peanut oil [1] weekly for 24 weeks (total dose, 1.2 mg.). Ten animals received 0.05 mg. of stilbestrol [2] in 0.25 cc. of sesame oil weekly for 5 weeks; at this time 5 mice were continued on the same dose for 19 additional weeks (total dose, 1.2 mg.), while for the other 5 the dose was increased to 0.25 mg. of stilbestrol in 0.25 cc. of sesame oil for 19 weeks (total dose. 5.0 mg.). The third group of 10 females was kept as untreated controls.

[1] *Theelin, Parke-Davis.*

[2] *Procured from Prof. E. C. Dodds, through Dr. M. J. Shear.*

Twenty-five $C_3H$ males, from nine litters, were divided into three groups. Ten mice were given subcutaneous injections of 0.05 mg. of estrone in 0.25 cc. of purified peanut oil weekly for 24 weeks (total dose, 1.2 mg.). Five animals received 0.05 mg. of stilbestrol in 0.25 cc. of sesame oil for the same length of time (total dose, 1.2 mg.). Ten mice were injected with 0.05 mg. of stilbestrol weekly for 5 weeks, and with 0.25 mg. of the compound in 0.25 cc. of sesame oil for 19 additional weeks (total dose, 5.0 mg.). Twenty other $C_3H$ males were injected weekly with 0.25 cc. of purified peanut oil or of sesame oil for 24 weeks and were used as controls.

The mice were examined weekly for breast tumors. Some difficulty was encountered in distinguishing oil deposits from tumors, and two male mice, one receiving stilbestrol and the other estrone, were killed erroneously. In each case the mass proved to be encapsulated oil instead of tumor. As soon as a hard mass was palpable, the animal was marked and permitted to live for another week, when it was killed with ether or by cervical dislocation and autopsied immediately.

## RESULTS

Two female mice injected with the larger doses of stilbestrol died of pyometra on the eighteenth and twenty-first weeks of injection, having received 3.5 and 4.25 mg. of the compound, respectively. One male mouse injected with estrone died of pneumonia in the thirty-fourth week. The remainder of the animals injected with the estrogens survived until they either developed breast tumor or until the fifty-fourth week, when the experiment was terminated. Half of the control males injected with sesame or peanut oil were killed at this time, and half were killed 4 months later, when they were 17.5 months of age.

As indicated in table 1, which summarizes the results, all females which tolerated the course of injections developed breast tumors. Mammary tumors appeared in the untreated virgin controls at an average age of 46.5 weeks. When 1.2 mg. of stilbestrol was injected, the average latent period was reduced to 34.8 weeks, while the same dose of estrone reduced it to 30.3 weeks.

The impression that estrone is more potent than stilbestrol in accelerating the appearance of breast tumors in $C_3H$ females is accentuated when litter mates are considered. In every instance, the litter mate which received estrone developed a breast tumor earlier (by 1, 3, 5, 8, and 12 weeks) than its sister which was injected with stilbestrol.

TABLE 1.—*Carcinogenicity of stilbestrol and estrone, in $C_3H$ mice*

| Sex | Injection | Total dose | Number of mice used | Number of breast tumors in mice, at various ages (in weeks) | | | | | | | | | | | | | | | Total tumors | Average tumor age |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 24 | 26 | 28 | 30 | 32 | 34 | 36 | 38 | 40 | 42 | 44 | 46 | 48 | 50 | 52+ | | |
| | | *Mg.* | | | | | | | | | | | | | | | | | *Percent* | *Weeks* |
| F | Stilbestrol | 5.0 | 5 | | | 2 | | | | | | 1 | | | | | | | [1] 100 | 31.0 |
| F | do | 1.2 | 5 | | | | 1 | | 2 | 1 | | | 1 | | | | | | 100 | 34.8 |
| F | Estrone | 1.2 | 10 | 1 | 1 | | 3 | 1 | 2 | 2 | | | | | | | | | 100 | 30.3 |
| F | | | 10 | | | | | | 1 | | 1 | | | 1 | 1 | 1 | 2 | 3 | 100 | 46.5 |
| M | Stilbestrol | 5.0 | 10 | 1 | | | | 2 | 4 | | | | | | | | 1 | | 80 | 34.4 |
| M | do | 1.2 | 5 | | | | | | | | | | | | | | 1 | | 20 | 50.6 |
| M | Estrone | 1.2 | 10 | | | | | | | 1 | 1 | | | | | | | | 20 | 36.7 |
| M | Sesame oil | | 10 | | | | | | | | | | | | | | | | 0 | |
| M | Peanut oil | | 10 | | | | | | | | | | | | | | | | 0 | |

[1] 2 mice died of pyometra before tumors appeared in group.

Stilbestrol in sesame oil, estrone in peanut oil, injected subcutaneously weekly for 24 weeks.

With one exception, in which an untreated female developed a breast tumor 4 weeks after the litter mate which received estrone but 7 weeks before the litter mate injected with stilbestrol, the breast tumors appeared from 7 to 27 weeks later in the untreated females than in the litter mates which received the estrogens. There was no correlation, however, between the susceptibility of the litter mates to tumor induction with estrogens and to the development of spontaneous breast tumors, i. e., the untreated litter mates of the females in which tumors appeared earliest with estrogen stimulation did not develop spontaneous breast tumors at the earliest age, and vice versa.

Eight of the ten males which received 5.0 mg. of stilbestrol developed breast tumors. At the comparable dose of 1.2 mg., the latent period of one tumor which appeared in a mouse treated with stilbestrol was 13 weeks longer than the latent period of the two tumors which developed in the mice receiving estrone.

Three mice, a female treated with estrone and a male and a female injected with stilbestrol, had two mammary tumors each. The low incidence of multiple tumors was undoubtedly owing to the fact that the animals were killed as soon as a definite tumor was palpable, instead of being retained until natural death.

The different solvents employed for the two estrogens introduces an unevaluated factor which may have an effect on the results. There was a marked difference in the response of the animals to the two solvents. Sesame oil apparently was absorbed, as the mice looked normal and little of the substance was present subcutaneously a month after the last adminis-

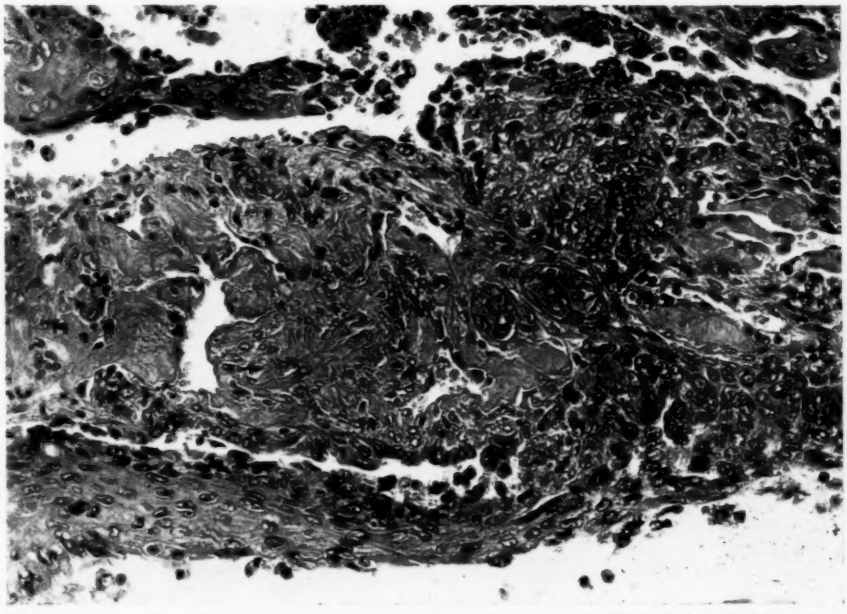


FIGURE 1.—An area of squamous carcinoma containing large masses of keratin in a breast tumor. Haematoxylin and eosin. ×360.

249431—40——

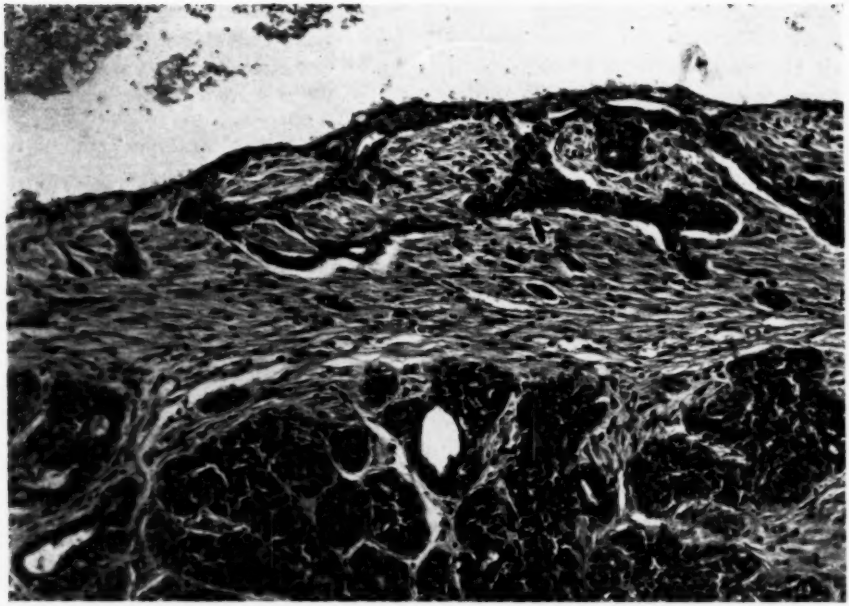


FIGURE 2.—Section from a breast tumor of a $C_3H$ female which received 5.0 mg. of stilbestrol. Note the abundant cellular stroma and tumor cells growing in thin columns. Haematoxylin and eosin. ×205.

tration. Peanut oil was tolerated poorly in that it was not absorbed and produced huge oil deposits, local tissue reaction, and abscesses which did not recede for as long as 11 months after the last injection.

## HISTOLOGIC OBSERVATIONS

In all cases the thyroid, adrenals, tumors, or tissue at the site of injection were examined. The testes, seminal vesicles, prostate, and penis of males were fixed and sectioned; while the ovaries and tubes, uterine horns, cervix, and vagina of females were examined.

### BREAST TUMORS

From a histologic standpoint there was no significant difference in the character of the tumors occurring in the stilbestrol group as compared with those observed in the estrone-treated mice except for the occurrence of small areas of squamous carcinoma in four mice receiving estrone (fig. 1). No such changes were seen in the stilbestrol group. Three of the four tumors containing squamous areas arose in female mice. The epithelial elements were usually grouped in broad alveoli or nests of acini lined by cuboidal tumor cells in which mitoses were frequent. In many cases where the stroma was abundant, the tumor cells appeared compressed in slender columns or strands (fig. 2). The stroma in 27 of the 29 breast tumors arising in treated mice was far more abundant and active than is usual in the spontaneous breast tumors in mice of this strain. The stroma usually contained many elongated fibroblasts set in a network of collagen fibers which frequently were slightly separated and occasionally appeared edematous. Capillaries were numerous and well formed. Hemorrhagic areas were seen in

approximately half of the tumors in treated mice. They were smaller than those noted in spontaneous tumors allowed to grow until the death of the mouse.

No tumors other than those of the breast were found in the male or female mice injected with the estrogens. In the control $C_3H$ male group injected with the oils, one mouse had a small hepatoma at 54 weeks, when the animals were about 14 months of age. Nine males which had received peanut or sesame oil injections were killed 18 weeks later, when they were 17.5 months of age. Of these, three had hepatomas and two had single pulmonary tumors.

### UTERINE HORNS

Pyometra occurred in two mice. The endometrial cavities were dilated and filled with an exudate consisting of amorphous debris, fibrin, and large numbers of polymorphonuclear leukocytes in varying stages of disintegration. The endometrial lining had largely sloughed away and was replaced by large macrophages with foamy, vacuolated cytoplasm. The inflammatory process involved the endometrium and myometrium, and the lumens of many glands contained an inflammatory exudate similar to that in the endometrial cavity (fig. 3). In a few areas the glandular epithelium underwent a partial squamous metaplasia.

Varying degrees of hyalinization of the endometrial stroma were seen in all but three of the treated animals. The process was more extensive in the number as well as in the size of the individual foci in mice receiving larger doses of stilbestrol, and approximately equal in those receiving

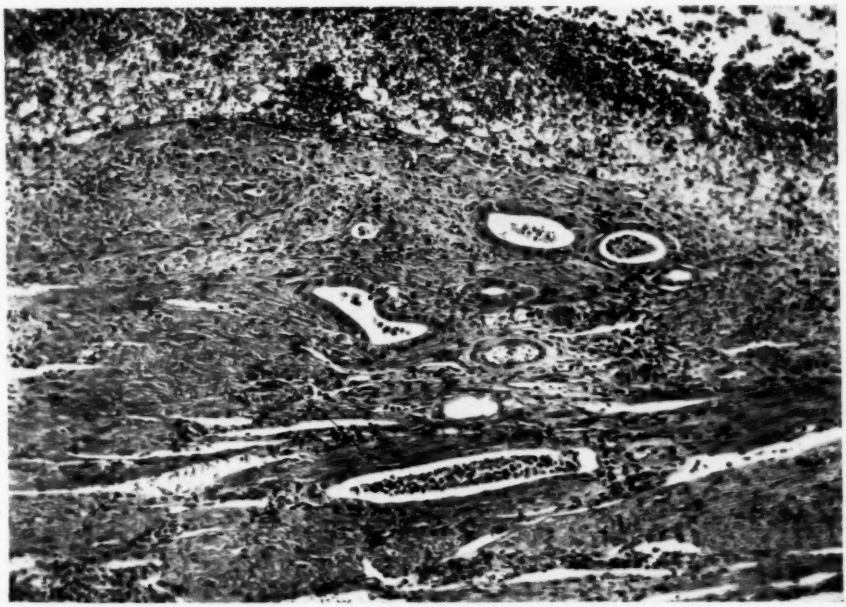


FIGURE 3.—Section of the uterine wall in a case of pyometra. The inflammatory exudate involves not only the cavity at the upper portion of the field, but also all the coats of the uterine wall. Haematoxylin and eosin. ×140.

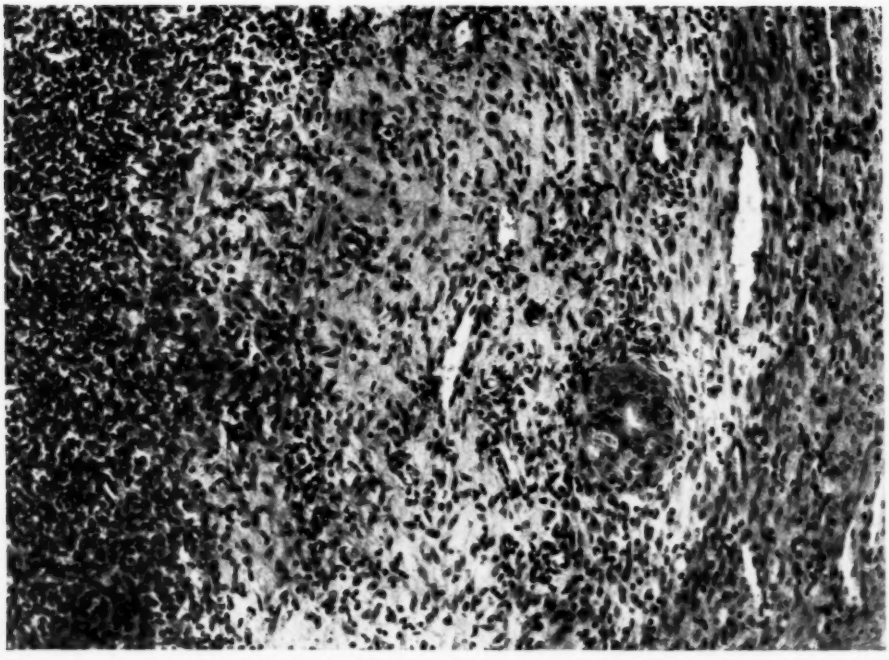


FIGURE 4.—An area of hyalinization in the endometrial stroma. Note the sharp demarcation of the lesion at the myometrium. Haematoxylin and eosin. ×195.

smaller doses of stilbestrol or estrone. In the more advanced stages the lesions appeared as moderately well circumscribed areas containing a pale, smooth, and usually structureless substance in which were scattered varying numbers of elongated nuclei resembling those of young fibroblasts. There were also smaller numbers of rounded, darkly staining lymphoid cells (fig. 4). In several of the smaller areas the ground substance appeared faintly fibrillar and with van Gieson's connective-tissue stain took on a faint reddish tinge. These findings together with negative results with the methyl violet stain for amyloid strongly suggest that the substance is collagenous in nature (8).

The lining epithelium of the endometrium and its glands was uniformly active in all the treated mice as evidenced by the large number of mitotic figures. In four animals (two of the high stilbestrol, one of the low stilbestrol and one of the estrone series) the endometrial lining was of a pseudostratified columnar type, but in the remaining animals it was of the simple columnar variety. Squamous metaplasia was not observed except in the mice with pyometra, and there was no suggestion of any invasive properties on the part of the endometrial lining.

### CERVIX AND VAGINA

No tumors or definitely invasive lesions of the cervix or vagina were observed in any of the three groups of treated mice. In both the stilbestrol- and estrone-treated mice the epithelium was hyper-

plastic, well-stratified, and usually heavily keratinized when compared with that in the untreated animals. Mitotic figures were numerous, and there were many well-defined prickle cells. The cells of the basal layers were usually hyperchromatic, and frequently some disturbance of polarity was noted (figs. 5 and 6). The cervical and vaginal stroma in the treated animals was usually edematous; and in two of the mice receiving large doses of stilbestrol there were large hyaline areas similar to those noted in the uterine horns.

OVARIES

The ovaries of the treated mice were uniformly active although not appreciably more so than in the control group. In two mice of the high stilbestrol group small follicular cysts were seen on microscopic examination.

MALE GENITALIA

In the testes of all the treated mice the tubular epithelium was well-preserved, and spermatogenesis appeared to be proceeding actively. The interstitial cells were entirely normal in number and appearance.

No changes were noted in the mucosa of the seminal vesicles, prostates, or penises of the treated mice. Grossly the seminal vesicles appeared larger than those in the control groups, but this difference was not clearly demonstrable in histologic sections.

ADRENALS

In both males and females of the treated groups there was a moderate, though definite, increase in the number of small, darkly staining oval cells of the cortex. They were most numerous in the zona glomerulosa, although they occasionally

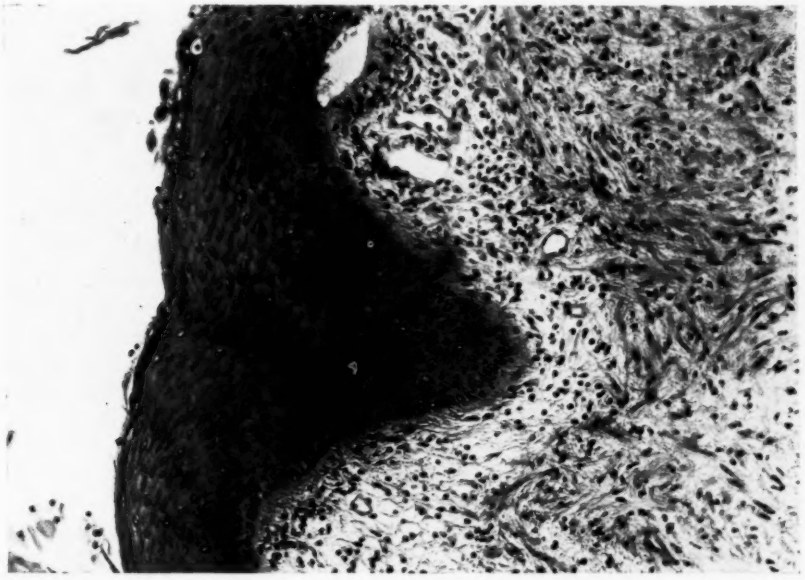


FIGURE 5.—The vaginal mucosa of a mouse which received 5.0 mg. of stilbestrol. Note the hyperplasia of the mucosa and the absence of invasiveness. Haematoxylin and eosin. ×205.

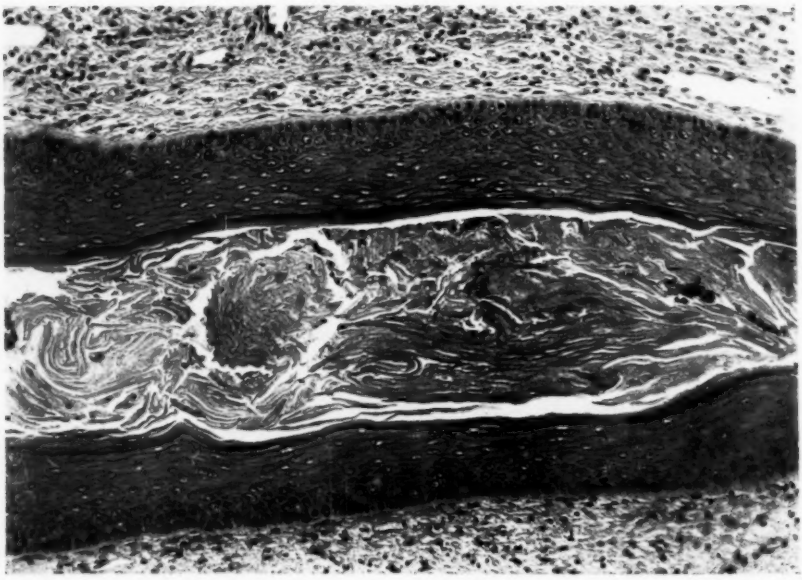


FIGURE 6.—The cervical canal from a mouse which received 5.0 mg. of stilbestrol. The mucosa is hyperplastic, and the cervical canal is dilated and filled with a mass of keratin. Haematoxylin and eosin. ×210.

infiltrated through the fasciculata as far as the zona reticularis.

#### THYROIDS AND LYMPHOID TISSUE

Examination of the thyroid gland failed to reveal any significant changes in any of the treated mice.

No changes which might be regarded as neoplastic were observed in the lymphoid structures of these animals. In several instances in mice which bore breast tumors, either spontaneous or induced, the spleens were slightly enlarged and showed moderate degrees of myeloid metaplasia. The subcutaneous lymph nodes, particularly those near the injection sites were frequently enlarged. Microscopically the medullary areas and sometimes the cortical follicles were extensively infiltrated with macrophages containing large droplets of brownish pigment granules within their cytoplasm. This process apparently was secondary to the repeated injections of peanut oil solutions which frequently formed chocolate-colored cysts in the subcutaneous tissues.

## DISCUSSION AND SUMMARY

The influence of various estrogens on the formation of breast tumors in mice appears to be proportional to their estrogenic activity (*3*). Parenterally, stilbestrol is stated to be two-and-a-half times as potent as estrone by the vaginal-smear assay on ovariectomized rats (*9*), and more active than estrone in man (*10*). In mice, however, Leighty and Wick (*11*) report that estrus is induced with 0.05 gamma of estrone and with 0.066 gamma of stilbestrol, and Perry (*12*) substantiates this estrogenic dose of stilbestrol. Emmens (*13*), using a somewhat different technique,

finds 0.1 gamma to be the mouse unit for both estrogens given subcutaneously.[3]

The carcinogenic potency of the two compounds in mice, therefore, is parallel to their estrogenic activity.

To elicit breast tumors in 20 percent of male mice of a highly inbred strain in which practically 100 percent of the females develop spontaneous breast carcinoma, 1.2 mg. of stilbestrol or estrone were given over a 6-month period, or over about one-fourth of the life span of the animals. The weekly doses of 0.05 mg. each were 500 to 1,000 times the estrogenic dose for mice, and the total doses were equivalent to at least 12,000 mouse-units per animal, or 400,000 units per kilogram.

The mice tolerated well the large doses of the two estrogens, and no marked lesions, except the development of breast tumors in both male and female mice and of the genital tract in females, were encountered. Gardner and Allen (*14*) reported the occurrence of 10 cervical lesions in 76 $C_3H$ mice studied at an average age of 339 days after prolonged injections of estrogens. One of these animals had a metastasizing, transplantable carcinoma of the cervix. In this series of 20 $C_3H$ females, all showed hyperplasia of cervical and vaginal mucosa, but no invasive lesions were present. No lesions of the lymphoid apparatus suggestive of lymphoid tumors, as described by Gardner, Kirschbaum, and Strong (*15*) in 22 out of 149 $C_3H$ mice receiving estrogens, were noted in the 45 $C_3H$ mice injected with estrone or stilbestrol. Enlargement of the spleen and subcutaneous lymph nodes in these mice was certainly not of neoplastic origin. Splenic enlargement occurred in mice bearing breast tumors and was due to varying degrees of myeloid metaplasia, which is a frequent finding in tumor-bearing mice. The enlargement of lymph nodes appeared to be due solely to proliferation of macrophages evoked by the injections of oily solutions.

The investigation shows that (1) stilbestrol possesses the property common to all estrogens of eliciting mammary carcinoma in mice of susceptible strains; (2) weekly subcutaneous injections of 0.05 mg. of stilbestrol in 0.25 cc. of sesame oil for 6 months are well tolerated by male and female mice of strain $C_3H$, and no toxic manifestations or morphologic changes, except in the genital and breast tissues are noted; and (3) subcutaneously injected stilbestrol in sesame oil is slightly less potent in eliciting breast tumors in $C_3H$ mice than the same amount of estrone in peanut oil.

---

[3] *The molecular weights of the compounds are 342 for estrone ($C_{18}H_{22}O_2$) and 308 for stilbestrol ($C_{18}H_{20}O_2$), so that at identical weight doses actually 10 percent more stilbestrol is administered.*

## REFERENCES


(1) Dodds, E. C., Goldberg, L., Lawson, W., and Robinson, R.: Oestrogenic activity of certain synthetic compounds. Nature, London, **141:** 247 (1938).

(2) Council on Pharmacy and Chemistry: Stilbestrol. J. A. M. A., **113:** 2312 (1939).

(3) Gardner, W. U.: Estrogens in carcinogenesis. Arch. Path., **27:** 138 (1939).

(4) Lacassagne, A.: Apparition d'adénocarcinomes mammaires chez des souris males traitées par une substance oestrogène synthétique. Compt. rend. Soc. biol., **129:** 641 (1938).

(5) Geschickter, C. F.: Mammary carcinoma in rat with metastases induced by estrogen. Science, **89:** 35 (1939).

(6) Andervont, H. B., and McEleney, W. J.: Influence of non-breeding and foster-nursing upon the occurrence of spontaneous breast tumors in strain $C_3H$ mice. Pub. Health Rep., **53:** 777 (1938).

(7) Andervont, H. B.: Occurrence of spontaneous and induced pulmonary and liver tumors in strain $C_3H$ mice. Pub. Health Rep., **54:** 1158 (1939).

(8) Suntzeff, V., Babcock, R. S., and Loeb, L.: Reversibility of hyalinization in the mouse uterus produced by injections of estrogen. Am. J. Cancer, **38:** 217 (1940).

(9) Dodds, E. C., Lawson, W., and Noble, R. L.: Biological effects of synthetic oestrogenic substance 4:4′-dihydroxy-$\alpha$:$\beta$ diethylstilboene. Lancet, **1:** 1389 (1938).

(10) Shorr, E., Robinson, F. H., and Papanicolaou, G. N.: Clinical study of the synthetic estrogen stilbestrol. J. A. M. A., **113:** 2312 (1939).

(11) Leighty, J. A., and Wick, H. J.: Diethylstilbestrol compared to estrone in causing estrus in spayed mice. Endocrinology, **25:** 597 (1939).

(12) Perry, I. H.: Seventh Scientific Report. Internat. Cancer Research Foundation, p. 105, Philadelphia (1939).

(13) Emmens, C. W.: Duration of action of certain natural and synthetic oestrogens when administered orally or by injection. J. Endocrinol., **1:** 142 (1939).

(14) Gardner, W. U., and Allen, E.: Malignant and non-malignant uterine and vaginal lesions in mice receiving estrogens and estrogens and androgens simultaneously. Yale J. Biol. Med., **12:** 213 (1939).

(15) Gardner, W. U., Kirschbaum, A., and Strong, L. C.: Lymphoid tumors in mice receiving estrogens. Arch. Path., **29:** 1 (1940).